

STUDIES ON BACTERIA ABLE TO USE

METHANE IN DENITRIFICATION

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M.Sc.

by

TREVOR RODNEY DAVIES

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S U M M A R Y

The aim of this investigation was to determine the suitability of single carbon organic compounds as hydrogen donors for denitrification. Although methanol has been used for this purpose with some success, methane has apparently been disregarded as a possible carbon source.

A denitrifying unit was operated with methanol as a carbon source, in order to determine the influence of methanol on rates of denitrification with several different nitrate concentrations. With methanol as an additive, nitrate reduction was of the order of 90%, while less than 40% denitrification was achieved with the supernatant from settled domestic sewage as the sole carbon source.

Isolates were taken from this unit for identification.

A similar unit was constructed, receiving methane as the sole carbon source. Bacteria were isolated from this unit and shown to be able to use nitrate as a terminal electron acceptor and as a source of cell nitrogen. These bacteria were identified and their specificity to methane tested. In addition to methane they were found to be able to use several other organic compounds for growth and denitrification.

Isolates from the methanol unit were found to be able to use methane for denitrification.

Alcaligenes was the predominant genus among methane oxidising bacteria isolated from both the methane and methanol denitrification units.

None of the isolates from either unit was able to use molecular hydrogen to support growth.

The production of  $N_2O$  during denitrification was dependent on both the strain of bacteria concerned and the nature of the carbon source. Hydrogen and  $N_2O$  production did not occur simultaneously.

The optimum pH range for denitrification by methane oxidising bacteria was between 7,0 and 8,5. The bacteria investigated operated most efficiently at  $30^{\circ}C$  when grown on methanol or lactate and between  $20^{\circ}C$  and  $30^{\circ}C$  when grown on methane.

A packed column denitrifying unit receiving methane as the sole carbon source removed 50% of nitrate nitrogen from a nitrified effluent.

Methane denitrification was proposed as a commercially attractive method of removing low concentrations of nitrate from large volumes of water.

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## I. INTRODUCTION

### 1. NEED FOR INVESTIGATION

In recent years the necessity for removing plant nutrients such as nitrogen from water has become steadily more pressing, due to two major considerations. Water produced by sewage treatment plants must have all or most of the nitrogen removed from it, in order to prevent the establishment of eutrophic conditions in lakes and dams receiving this water. Dry countries, like South Africa, cannot afford to waste any water. This fact has led to the development of schemes for the direct renovation of sewage and other polluted waters to the standard of potable waters, a process generally known as water reclamation. The schemes already in operation rely on purely physical-chemical stripping of ammonia from the water. Unfortunately only ammonia is removed by such treatment and no provision is made for the removal of other nitrogenous compounds.

The combination of biological nitrification and denitrification has been suggested as an alternative method of nitrogen removal. Nitrification has received a good deal of attention, as in the past water effluent standards called for all dissolved nitrogen to be in the nitrate form. Denitrification has already been applied, to some extent, to agricultural wastes and pilot plant studies have been made on treatment of industrial and domestic waste waters, but generally these have been approached as purely engineering problems. Some of these investigations have involved the use of methanol, but little attempt has been made to gather knowledge about the bacteria utilising methanol as a hydrogen donor in denitrification. In spite of its ready availability at sewage treatment plants, methane has largely been overlooked as a carbon source. This is probably due to the fact that methane-oxidising bacteria have been widely

reported as carrying out methane oxidation only in the presence of molecular oxygen, being unable to substitute nitrate as the terminal electron acceptor.

It is important to know if a special population is needed for denitrification with single carbon hydrogen donors, or if a population grown on a different carbon source, such as sewage, could adapt to a rather specific substrate such as methanol or methane.

If methane oxidising bacteria were found to denitrify with carbon sources other than methane, it would be necessary to gain some knowledge about the effect of environmental conditions on their growth and denitrification rates before the system could be used commercially. As temperature and pH are two of the most important environmental factors affecting the growth and development of microorganisms, it would be of advantage to examine the effect of these two factors on methane-utilising denitrifying bacteria grown on different carbon sources.

## 2. SPECIFIC OBJECTIVES

The specific objectives of this investigation are:

- (1) Isolation and identification of bacteria able to use methanol as a hydrogen donor for denitrification.
- (2) Assessment of their ability to use methane as a hydrogen donor.
- (3) Isolation and identification of bacteria able to use methane as a hydrogen donor for denitrification.
- (4) Assessment of the specificity of the above bacteria to single carbon compounds as hydrogen donors.
- (5) Development of a chemostat for the study of pure cultures of bacteria under various growth conditions.

- (6) Growth of bacteria able to make use of nitrate respiration during methane oxidation, on methane, methanol or lactate at various pH values.
- (7) A repetition of the above experiment with the pH constant, but at several different temperatures.

## II. LITERATURE SURVEY

### 1. METHODS OF NITROGEN REMOVAL

#### (i) Standard physical-chemical procedure

Chemical schemes for water reclamation (Van Vuuren et al., 1967; Stander and Van Vuuren, 1969) depend on physical-chemical stripping of ammonia from the treated water. This involves raising the pH of the water to 11,0 - 11,2 and pumping it up a tower, from where it is allowed to trickle down a series of wooden slats, between which air is blown by large fans. The ammonia then passes into the atmosphere, where it remains a potential source of pollution. Such a system has several drawbacks, among which are the large capital outlay, a tendency to freeze up in winter, scaling caused by lime added to raise the pH, etc.

Because of the high pH at which the unit is operated, no nitrate is formed by nitrification during this process. Any nitrate which may already be in the solution is not removed, however, as the system is only capable of handling nitrogen in the ammonia form. Residual nitrate is brought below the statutory level of  $10 \text{ mg l}^{-1}$  by dilution with clean water, which makes continuous recirculation of the same volume of water impossible as long as ammonia stripping is used as the sole means of removing dissolved nitrogen compounds from water.

#### (ii) Means of biological nitrogen removal

##### (a) Incorporation

This method would rely on the growth of bacteria, yeasts, or any other organisms, in order to incorporate dissolved nitrogen compounds into cell material by assimilation of the

nitrogen compounds. Unless these cells are harvested, a costly process, this would only change the form of nitrogen and not remove it from the water.

(b) Incorporation and physical removal

This method is closely linked to the previous one. Here use is made of the photosynthetic capacity of algae to assimilate various forms of dissolved nitrogen compounds, followed by physical removal of the cells. It has the advantage of using energy obtained by photosynthesis for nitrogen removal, making chemical additives unnecessary and keeping operating costs low. This is not totally to the advantage of the system, however, as no ammonia is incorporated into cellular material during the hours of darkness.

St. Amant and Beck (1970) found that the most efficient depth for algal growth ponds is only about 40 cm, which would mean large areas of land being set aside for nitrogen removal. Ponds have a further disadvantage in the difficulty experienced in harvesting algal cells. Hemens and Stander (1969) overcame this problem by using a system of rotating discs bearing algal films. They found however, that most nitrogen removal was achieved, not by assimilation but by algal photosynthesis raising the pH to a point where ammonia readily came out of solution. They concluded that, despite its limitations, it would be more efficient to use a purely physical-chemical system to achieve the same ammonia stripping.

(c) Biological conversion to nitrogen gas

The biological conversion of ammonia into atmospheric nitrogen has gained considerable importance because the process

involves a nitrogenous end product which is not a pollutant but the major constituent of the atmosphere. Since all of the nitrogen present should be in the oxidised form for efficient conversion into molecular nitrogen, this biological treatment is a procedure involving two stages.

The first step is aerobic, ammonium ions being oxidised to nitrate by autotrophic nitrifying bacteria, typified by the genera Nitrosomonas and Nitrobacter. The next stage is anaerobic, inducing the denitrifying bacteria to use nitrate in place of oxygen as the terminal electron acceptor. This causes the nitrate to be reduced and converted into nitrogen in molecular form.

The system is continuous, being free of limitations imposed by light fluctuations, and relatively compact, saving on space. St. Amant and Beck (1970) found that a filter type denitrifying unit would occupy only 2% of the land needed for algal growth ponds. Such a filter or packed column unit offers several advantages over totally mixed algal or bacterial systems. The large solid surface area provided in a packed column unit would induce adsorption of low concentrations of nitrate and carbon compounds, concentrating them for bacterial action (Zobell and Grant, 1943). The column packing material would also provide for the attachment of denitrifying bacteria, allowing the use of hydraulic residence times of shorter duration than the minimum doubling time of the bacteria involved (Du Toit and Davies, 1972). Davies (unpublished results) has operated such a unit at less than a one hour hydraulic residence time and achieved over 80% denitrification with water containing  $100 \text{ mg l}^{-1}$  nitrate nitrogen.

Denitrification does have certain disadvantages, among which are:

- (1) Efficiency decreases with temperature.
- (2) An organic carbon source must be supplied as a hydrogen donor, causing increased costs.
- (3) The water must be monitored to ensure addition of organic carbon in the correct C:N ratio.
- (4) Cells produced must be removed from the effluent. This problem is reduced by the use of a packed column unit, but then provision must be made for the prevention of clogging.

Despite the limitations of the system the combination of bacterial nitrification and denitrification appears to offer the best prospect of efficiently removing nitrogen from large volumes of water under controlled conditions.

## 2. BIOLOGICAL REMOVAL OF NITROGEN BY NITRIFICATION-DENITRIFICATION

### (i) Outline of the procedures involved

#### (a) Nitrification

Since nitrification and denitrification are essentially two independent parts of a single system for removing eutrophying nitrogen from water, each process must be considered separately.

Five genera are listed in Bergey (1957) as being capable of oxidising ammonia to nitrite, namely Nitrosomonas, Nitrosococcus, Nitrosocystis, Nitrosospira and Nitrosogloea, but the last three named genera are of doubtful validity (Painter, 1970). Two genera of the bacteria are accepted as being capable of oxidising nitrite to nitrate. They are Nitrobacter and Nitrocystis. Other genera have been described,

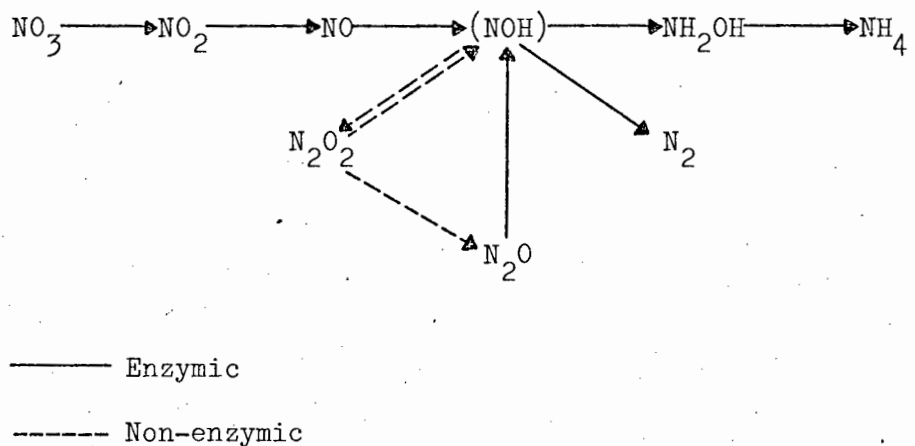


but their classification remains questionable. These oxidations are accepted to be exothermic reactions which supply the energy for the strongly endergonic synthesis of cell material from carbon dioxide (Nicholas, 1963). The energy metabolism of autotrophs, however, may resemble that of heterotrophs more closely than has previously been suspected (King, 1966). He concluded that Nitrobacter reduced NAD to  $\text{NADH}_2$ , using nitrite as the electron donor and ATP to provide the energy.  $\text{NADH}_2$  was then oxidised by orthodox electron transport to generate more ATP than originally consumed, by means of oxidative phosphorylation. Some  $\text{NADH}_2$  was still available for  $\text{CO}_2$  reduction.

This chemoautotrophic metabolism takes place relatively slowly and it is possible that it could exert a limiting effect on the overall process of biological nitrogen removal by restricting the supply of nitrate to the relatively fast growing denitrifying bacteria.

(b) Denitrification

Nitrate can be reduced in two ways, by assimilation and dissimilation. Fewson and Nicholas (1961a) have represented this as follows:



Nitrate assimilation involves the reduction of nitrate in order to incorporate nitrate nitrogen into cell material. Nitrate dissimilation, or nitrate respiration, is a process in which nitrate merely serves as a terminal electron acceptor in place of oxygen. Denitrification may be regarded as a special case of nitrate reduction, in which nitrogen gas or its oxides is produced from nitrate, nitrite or any other suitable oxidised intermediate (Nicholas, 1963).

Dawson and Murphy (1972) have stated that denitrifying bacteria are capable of converting nitrate to ammonia for cell growth, simultaneously with denitrification. Any similarity between the pathways for nitrate dissimilation and assimilation would increase the possibility of bacteria able to use nitrate for assimilation also being able to use it for respiration.

Several reviews have appeared dealing with various biochemical aspects of nitrate metabolism (Nason and Takahashi, 1958; Nason, 1962; Nicholas, 1963; King, 1966) but it should suffice to report that there does appear to be some relationship between the pathways of nitrate dissimilation and assimilation. Although Hofman and Lees (1953) found that the hydroxylamine was an intermediate in ammonia assimilation by Nitrosomonas, Hall and MacVicar (1955) could not detect a pathway involving hydroxylamine and oxime during formation of amino acids from nitrate. They felt that the evidence weighed strongly in favour of ammonia and the  $\alpha$ -imino acids as intermediates, by means of a mechanism similar, or even identical, to the nitrogen fixation pathway used by Clostridium.

If the mechanism is as Hall and MacVicar suggested, bacteria able to assimilate nitrate would only need an enzyme to convert nitroxyl into nitrogen in order to achieve denitrifying ability.

(ii) The need to separate the processes

Sewage purification installations such as the Huisman unit have been designed to achieve both nitrification and denitrification in the same tank. Although relatively good rates of nitrogen removal have been obtained through manipulation of this system, the vigour with which the water is mixed in the process makes optimisation of two essentially incompatible metabolisms a difficult task. Nitrifying bacteria are aerobic autotrophs, while denitrifying bacteria are facultative anaerobic heterotrophs. Skerman and MacRae (1957) reported that Pseudomonas denitrificans ceased denitrifying with oxygen concentrations as low as  $0,2 \text{ mg l}^{-1}$ . Working with Micrococcus denitrificans, Chang and Morris (1962) found that oxygen affects dissimilatory nitrate reduction in three ways:

- (a) Prevention of adaptive formation of necessary enzymes.
- (b) Repression of further dissimilatory enzyme synthesis.
- (c) Inhibition of preformed enzymes.

Smith (1970) and Mulbarger (1970) were able to achieve denitrification at dissolved oxygen concentrations of  $1,0 \text{ mg l}^{-1}$  due to localized oxygen depletion within sludge floc particles. These particles make possible the development of microhabitats so that both nitrifying and denitrifying bacteria can flourish in suitable conditions.

A further obstacle to the use of a mixed system arises out of the fact that denitrifying bacteria require organic carbon for growth and as electron donors in denitrification, while there is some evidence that organic compounds may inhibit nitrification (Lenhoff et al., 1965). Smith and Hoare (1968) found that Nitrobacter can grow on acetate by means of the Krebs cycle, without making use of either nitrite or  $\text{CO}_2$ . Wallace and Nicholas (1969) termed such bacteria "facultative autotrophs". It has also been discovered that bacteria undergo changes in their cytoplasmic membrane structures when they adapt from an autotrophic to a heterotrophic metabolism (Wang and Lungren, 1969). This could mean that it would take some time to revert back to autotrophic growth.

King (1966) has reported that, under anaerobic conditions, Nitrobacter can reverse its normal reaction and reduce nitrate to nitrite with  $\text{NADH}_2$  as the electron donor by a mechanism similar to the nitrate respiration of heterotrophic bacteria.

Clearly nitrification and denitrification cannot be accomplished efficiently together and the two processes must be separated for optimisation.

### 3. THE EFFECT OF THE CARBON SOURCE ON DENITRIFICATION

#### (i) The importance of the nature of the carbon source

Several investigators have applied denitrification to water treatment processes, but many of these workers have used the carbon compounds already present in untreated sewage as a source of hydrogen donors (Wuhrmann, 1964; Johnson and Schroepfer, 1964; Barth et al., 1967; Balakrishnan and Eckenfelder, 1970). These were essentially studies of different methods of recycling and settling. St. Amant and McCarty (1969), Seidel and Crites (1970) and Balakrishnan and

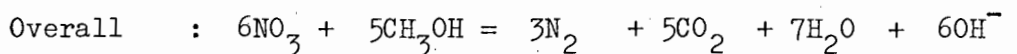
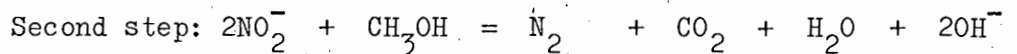
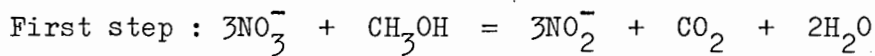
Eckenfelder (1970) all have suggested that an extraneous source of carbon must be supplied before total denitrification can take place. Although Wuhrmann (1964) has stated that any substrate normally used for respiration by the cells concerned may serve as an electron donor for nitrate reduction, it does not follow that all carbon sources which can be used are utilised with equal efficiency. Eckenfelder and Balakrishnan (1968) found that simple compounds, such as glucose, were more effective as hydrogen donors to enhance denitrification than complex organics. Davies and Toerien (1971) reported that the substitution of malate for glucose as the sole carbon source supplied to a suspended growth denitrification unit, caused a dramatic population shift. This was accompanied by a rise in denitrification from 11,5% to 90,5% (Davies et al., 1971).

After nitrification in conventional sewage treatment systems, little readily biodegradable organic carbon remains. This and the maintenance of anaerobic conditions are the two major problems in commercial denitrification. Du Toit and Davies (1973) found that settled sewage was unsuitable as a hydrogen donor, because it introduced reduced nitrogen in the form of ammonia into the system. Both lactate and methanol produced satisfactory denitrification, but the population took some time to adapt to methanol. Finsen and Sampson (1959) have used sucrose, molasses and ethanol as hydrogen donors, while St. Amant and McCarty (1969) found a wide range of inexpensive organic compounds to be suitable, namely sugar, acetic acid, acetone, ethanol and the cheapest of all, methanol. This observation was also made by Seidel and Crites (1970). It is fortunate that methanol is available at low cost, as Placak and Ruchhoft (1947) found that the lower alcohols are primarily oxidised by denitrifying bacteria, rather than synthesised to cell material.

(ii) The C:N ratio necessary for complete denitrification

Generally denitrification can be considered a two step process, first producing nitrites from nitrates and secondly reducing nitrites all the way to molecular nitrogen. Although some bacteria can only perform one of these two steps, the majority of denitrifying bacteria convert both nitrate and nitrite to molecular nitrogen (Alexander, 1961).

The denitrification reaction with methanol would be:



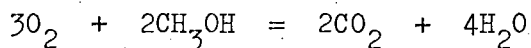
Therefore 1 mole of nitrate requires at least  $\frac{5}{6}$  mole of methanol for complete denitrification, or  $1,9 \text{ mg}\ell^{-1}$  of carbon as methanol for each  $\text{mg}\ell^{-1}$  of nitrate nitrogen. However Seidel and Crites (1970) have estimated that 25% of the methanol is used for cell synthesis. This would change the ratio as follows

$$\frac{1,9}{0,75}:1 = 2,5:1$$

This accords well with findings of Dawson and Murphy (1972), who considered that, although the theoretical carbon requirement for denitrification with glucose demanded a C:N ratio of 1,7:1, carbon removal during the logarithmic growth phase varied from 1,3 to 1,5 times the theoretical requirements. St. Amant and McCarty (1969) and Du Toit and Davies (1973) also derived a 2,5:1 ratio for methanol denitrification. The U.S. Federal Water Quality Administration (1970) arrived at a figure of between 2,5 and 3,0 to 1. St. Amant and Beck (1970) found that a 3:1 ratio was needed for 90% denitrification in both pond and filter applications, while Barth et al.

(1968) used a C:N ratio of 4:1.

Dissolved oxygen in a growth medium would use up part of the carbon source by normal respiration, reducing the efficiency of a denitrifying system. The excess methanol necessary to remove dissolved oxygen can be calculated as:



This would require 0,67 mg of methanol carbon to remove each mg of dissolved oxygen. Some denitrification systems have suffered so badly from the influence of dissolved oxygen that McCarty et al. (1969) found it necessary to add methanol at a C:N ratio of 30:1.

The total concentration of methanol required for complete denitrification can be calculated as follows:

$$C_m = 3,29N_o + 1,97N_1 + 1,16D_o$$

$C_m$  = required methanol concentration ( $mg\ell^{-1}$ )

$N_o$  = initial nitrate-nitrogen concentration ( $mg\ell^{-1}$ )

$N_1$  = initial nitrite-nitrogen concentration ( $mg\ell^{-1}$ )

$D_o$  = initial dissolved oxygen concentration ( $mg\ell^{-1}$ )

This equation is derived from that of St. Amant and Beck (1970) by dividing by a figure of 0,75, as suggested by Seidel and Crites (1970).

(iii) The mechanism for the utilisation of  $C_1$  compounds

Microorganisms capable of growth on single carbon compounds can be divided into groups as follows:

Group	Energy Source	Growth condition	Carbon source
A	inorganic oxidation, 'light	aerobic or anaerobic	CO <sub>2</sub>
B	organic oxidation	aerobic	reduced C <sub>1</sub> compounds
C	organic dismutation	anaerobic	CH <sub>3</sub> OH, HCO <sub>2</sub> H, CO <sub>2</sub>

(after Quayle, 1963).

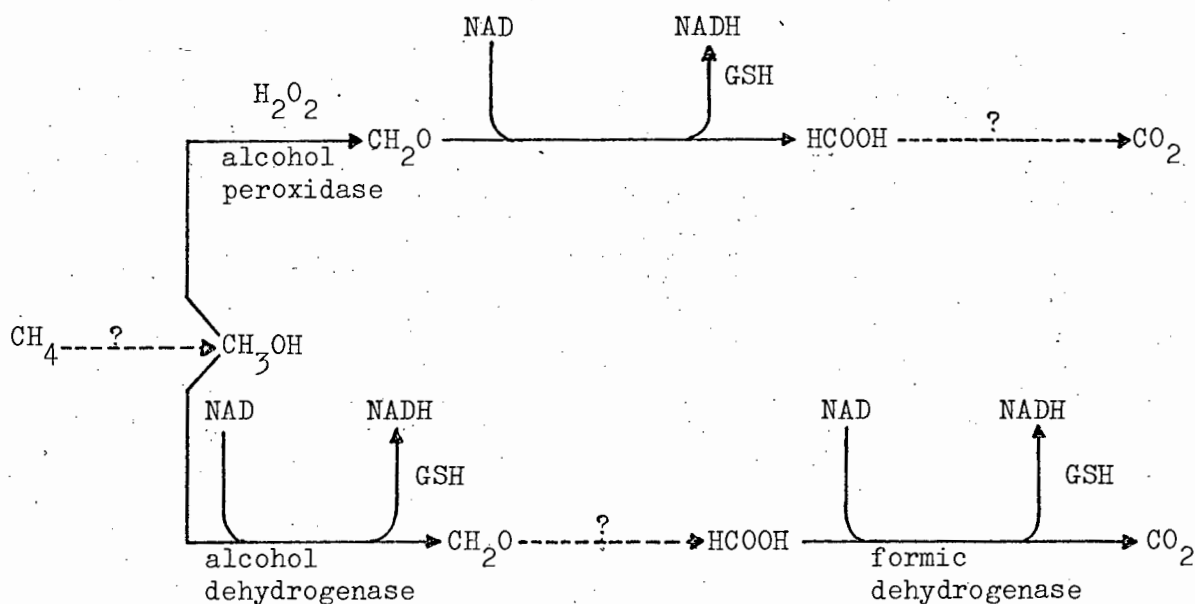
Many species of bacteria are capable of growth on C<sub>1</sub> compounds as the sole carbon and energy source. The mechanism whereby such organisms grow has long been the subject of speculation. It has been suggested (Bhat and Barker, 1948; Van Niel, 1954) that they might couple the energy of oxidation of the organic substrate to the reduction of CO<sub>2</sub> and the subsequent synthesis of CO<sub>2</sub> to cell material. This would be a variant of chemosynthetic autotrophy, in which the organic compound would merely play a role similar to the inorganic electron donor. An autotrophic metabolism of this type has been found in Pseudomonas oxalaticus, when grown on formate (Quayle and Keech, 1958; 1959a; 1959b).

Evidence of a heterotrophic kind of metabolism, involving the concomitant assimilation of substantial quantities of both CO<sub>2</sub> and C<sub>1</sub> units more reduced than CO<sub>2</sub>, has been found in several other organisms, including Pseudomonas methanica (Dworkin and Foster, 1956; Leadbetter and Foster, 1958), Pseudomonas PRL-W4 (Kaneda and Roxburgh, 1959) and Pseudomonas AM1 (Quayle and Peel, 1960), growing on methane, methanol or formate.



As methane would be available at little or no cost at sewage treatment plants, it is imperative that metabolism of both methane and methanol should be considered. Although there are no reports of methane being used for denitrification, it has been shown that Micrococcus denitrificans can use gaseous hydrogen for denitrification (Verhoeven, 1952; Verhoeven et al., 1954). This suggests the possibility that methane could serve as a hydrogen donor for denitrification.

The two alternative pathways for bacterial oxidation of methane and methanol have been presented by Silverman (1964) as:



Johnson and Quayle (1965) discovered that methane is oxidised via methanol, formaldehyde and formate and demonstrated the enzymes involved in this sequence in cell free extract of Pseudomonas methanica. They concluded that methane or methanol is assimilated heterotrophically at the reduction level between methanol and formate. The absence of both carboxydismutase and phosphoribulokinase during methane or methanol oxidation (Quayle and Keech, 1959; 1960; Large et al.,

1961) reinforced this assertion. Johnson and Quayle (1965) asserted there was small possibility of deviation from the described pathways.

#### 4. METHANE OXIDATION

##### (i) The feasibility of using methane for denitrification

If methane could be used as a hydrogen donor for denitrification it would have great economic significance. It is available at virtually no cost and, being a gas, it would eliminate the necessity for monitoring nitrate concentrations in sewage effluents in order to obtain the correct C:N ratio. It could share one of the advantages of methanol, which is that methanol is transformed into no compounds other than cell constituents and  $\text{CO}_2$  (Whittenbury et al., 1970).

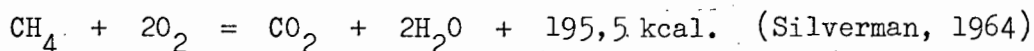
##### (a) The ability of methane oxidising bacteria to reduce nitrate

The lack of research in this direction is apparently due to discouraging reports about the ability of methane-oxidising bacteria to reduce nitrate, particularly by dissimilatory reduction. While Vary and Johnson (1967) were unable to find any isolates which could use nitrate even as a source of cell nitrogen, Leadbetter and Foster (1958), Dworkin and Foster (1956) and Whittenbury et al. (1970) all found that methane-oxidisers preferred nitrate to ammonium salts for cell synthesis. Whittenbury et al. (1970) did, however, report that their isolates were unable to grow anaerobically on methane with nitrate as an alternative electron acceptor to oxygen. It is probable, however, that denitrification is a fairly simple modification of the widespread ability to assimilate nitrate (Nicholas, 1963).

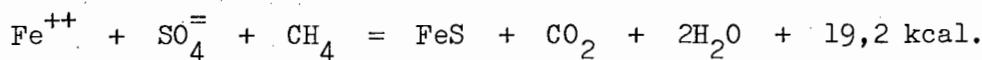
It seems likely that the process of nitrate assimilation follows the inorganic sequence to ammonia. Nitrate dissimilation appears to be merely an adaptation of the assimilatory pathways, partly by the intervention of cytochromes in the penultimate electron transfer sequence (Fewson and Nicholas, 1961a,b). It would appear unlikely that no bacteria exist which possess the mechanism to oxidise methane, using the process of nitrate respiration.

(b) Thermodynamic considerations

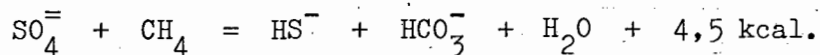
Previously isolated methane-oxidising bacteria were all aerobic, deriving their energy from the following exothermic reaction:



Sorokin (1957) failed to achieve growth of sulphate reducing bacteria on methane in the absence of oxygen. This is not altogether surprising, however, as the reaction would only be very weakly exothermic (Baas Becking, 1957):



and



It is also doubtful whether the sulphate reducers possess the enzymatic machinery to couple methane oxidation to sulphate reduction.

Nitrate respiration, however, yields practically as much energy as ordinary aerobic respiration (Stickland, 1931; Engel, 1958), which would mean that there would be neither enzymatic nor thermodynamic obstacles to denitrification with

methane as the hydrogen donor.

(ii) Specificity of methane-oxidising bacteria

There has been a great deal of controversy about the degree to which methane-oxidising bacteria are dependent on methane as a source of cellular carbon and energy. There is general agreement that they can use methanol, as organisms will generally grow at the expense of a group of carbon compounds closely related to the one employed to isolate the culture originally (Foster, 1962). Methanol can in fact be accepted as being the first stage in the oxidation of methane, followed by formaldehyde and formate (Johnson and Quayle, 1965).

Whittenbury et al. (1970) have stated that bacteria capable of using methane are unable to use carbon compounds other than methanol as alternative carbon sources. Leadbetter and Foster (1958) found that, although bacteria dependent on methane and methanol were capable of oxidising various alcohols, they could not use them to support growth. Foster and Davis (1966) had previously suggested the division of the bacteria in question into "obligate" and "facultative" methane and methanol users. These terms were also used by Vary and Johnson (1967), who found that some of their isolates were able to utilise methane, glucose and a series of alcohols. On the other hand, Dworkin and Foster (1956) reported that, although glucose stimulated the growth of Methanomonas methanica, methane was still an essential growth requirement. Perry (1968) investigated the specificity of bacteria to various compounds, including several gaseous hydrocarbons, and concluded that cells grown on gaseous alkanes readily oxidise all the normal alkanes in the series  $C_1-C_8$ , suggesting that the primary oxygenase for normal aliphatic

hydrocarbons does not have a high degree of specificity. He also emphasized that the solubility of n-alkanes in water is inversely proportional to their chain length. M. methanica was found to grow on glucose, lactose and malic acid and several other compounds as well as methane (Söhngen, 1906). He did not try methanol as a carbon source, but presumably this would have been suitable. Clearly enough uncertainty existed to suggest that denitrification with methane as the hydrogen donor might indeed be possible.

#### 5. PREVIOUSLY DESCRIBED DENITRIFYING BACTERIA

Denitrification is characteristic of a wide variety of common facultative bacteria, including the genera Pseudomonas, Achromobacter and Bacillus (St. Amant and McCarty, 1969). Typical denitrifying bacteria listed by Painter (1970) included the genera Micrococcus, Pseudomonas, Denitrobacillus, Spirillum, Bacillus and Achromobacter. Strains of Pseudomonas sp., Achromobacter sp., and Bacillus sp. capable of denitrification, have comprised as much as 51% of the organisms scraped from columns actively denitrifying agricultural wastewater (Smith et al., 1970).

Van Gils (1964) reported that Achromobacter, Alcaligenes and Flavobacter were predominant in both natural and laboratory activated sludge, while Pseudomonas occurred in relatively low numbers.

Davies and Toerien (1971) described an actively denitrifying population in which the major representatives were species of Achromobacter, Bacillus and Alcaligenes.

Methane-oxidising bacteria have been investigated by a wide range of workers (Silverman, 1964), but they seem to have limited their enquiries to a small number of isolates, including Pseudomonas methanica,

Methanomonas methanica, Methanomonas methanoxidans, Methanomonas carbonatophila and a species referred to as Bacterium methanicum by Münz (1915). There appears to be no further reference to this species, which Silverman's comparison shows to be similar to the strain of Methanomonas methanica used by Subbota (1947). However Dworkin and Foster (1956) suggested that Methanomonas methanica might be better named Pseudomonas methanica. The Bacterium methanicum described by Münz could then be regarded as a strain of Pseudomonas methanica.

Although none of these bacteria have been found to possess the ability to denitrify, they were bacteria isolated for their ability to utilise methane, without regard for their denitrifying capacity. Perhaps different results would have been forthcoming if known denitrifying bacteria had been tested for their ability to utilise methane. This was the technique used with success by Verhoeven (1952) when he found that molecular hydrogen could be used in denitrification.

Virtually all described methane-oxidising bacteria have proved to be Gram negative (Whittenbury et al., 1970) rods with flagella (Silverman, 1964). The exceptions are two species of Mycobacterium isolated by Nechaeva (1949).

## 6. THE EFFECT OF pH ON DENITRIFICATION

Since suspensions of denitrifying bacteria tend to become alkaline during the reduction of nitrate or nitrite to nitrogen, it is important to consider the effect of changing the pH on the denitrification rate. Karlсен (1938) found that Pseudomonas aeruginosa achieved notable denitrification over a range of pH 5,8 to 9,2, with an optimum activity between 7,0 and 8,2. Although Allen and Van Niel (1952) agreed that denitrification took place even above pH 9,0, they found that an isolate they referred to as Pseudomonas stutzeri showed optimum activity over a slightly

acid range, between pH 6,5 and 7,0.

Wijler and Delwiche (1956) reported that the gases evolved by mixed cultures varied with fluctuations in the pH. They found that at neutral or acid pH,  $N_2O$  was produced, but once the pH rose above 7,3 the  $N_2O$  was replaced by  $N_2$  as an end product.

Wuhrman and Mechsner (1965) found that at pH 5,5 to 6,5 aeration did not impair denitrification. At neutral pH, however, oxygen had a strongly inhibitory effect on denitrification.

## 7. THE EFFECT OF TEMPERATURE ON DENITRIFICATION

The seasonal variation in ambient temperatures make the effect of temperature one of the most important considerations for the operation of a commercial denitrifying system.

St. Amant and Beck (1970) suggested that a great drop in efficiency occurs if the temperature falls below  $10^{\circ}C$ . This has been borne out by R.J.L.C. Drews (personal communication) in studies on the Huisman unit.

Dawson and Murphy (1972) found that, although denitrification at  $5^{\circ}C$  was only 20% of that at  $20^{\circ}C$ , it was still considerable. Wheatland et al. (1959) also recorded denitrification at  $5^{\circ}C$ , but they found that at this temperature denitrification was only 10% of that achieved at  $25^{\circ}C$ . An U.S.A. Water Pollution Control Research Report (1970) concluded that denitrification rates were better at  $30^{\circ}C$  than at lower temperatures and that the C:N ratio required for denitrification rose as the temperature decreased.

### III. METHODS

#### 1. ISOLATION OF BACTERIA USING METHANOL AS A HYDROGEN DONOR FOR DENITRIFICATION

##### (i) Operation of a denitrifying unit receiving methanol as extraneous carbon source

###### (a) Description of the system

A perspex unit was constructed with a total volume of 11 litres (Fig 1). No packing was used for bacterial attachment. The unit was filled with liquid (see below) to an operating volume of 10 litres. Bacterial cells remained suspended in the culture fluid throughout the various stages of the experiment. Such a system can be referred to as a suspended growth unit. This kind of installation relies solely on the bacterial growth rate to maintain a sufficient microbial population.

The unit was sealed in order to keep the reactor virtually anaerobic. The suspension was mixed periodically by a centrifugal pump. Volume was controlled by a simple overflow system. The unit was operated in a constant temperature room at 20°C.

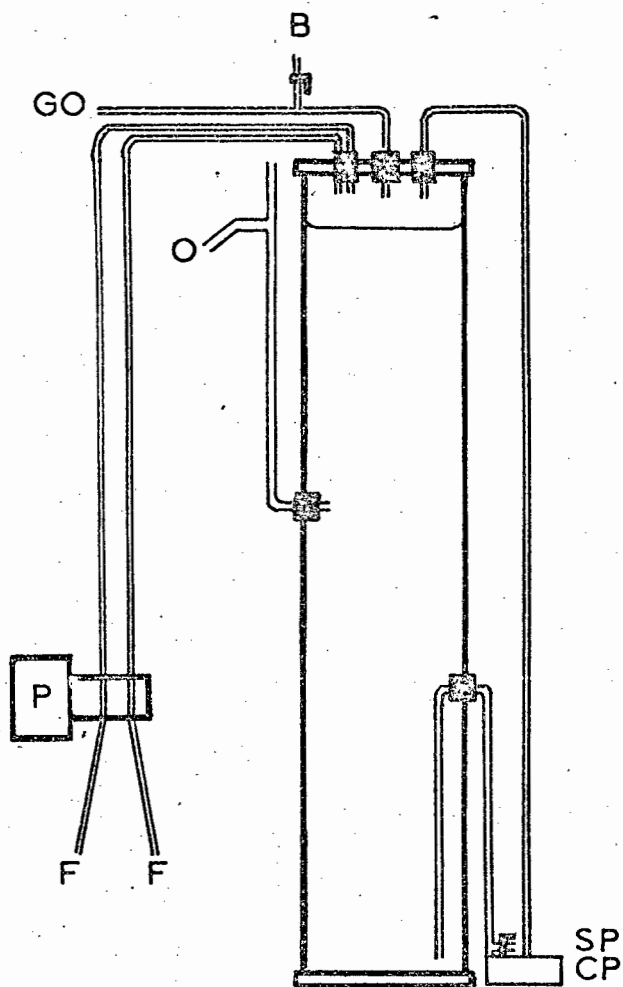
Samples of activated sludge and scrapings from the top of an anaerobic digester receiving domestic waste were mixed and inoculated into the denitrifying unit. In order to adapt the population, the unit was filled with equal quantities of a nitrate-methanol solution and the supernatant fluid from settled sewage and allowed to stand for several days. Feeding was then commenced using a model T8 Sigmapump (Middleport, N.Y.), allowing a hydraulic residence time of 40 hours. The C:N ratio was calculated from the equation proposed by St. Amant and McCarty (1969), taking dissolved oxygen into account.



FIGURE 1 : Suspended growth denitrification unit.

Legend for figure 1

P	Feed pump
F	feed line
GO	gas outlet
B	pressure equalisation bladder
SP	sample point
CP	circulation pump
O	medium overflow



The composition of the feed used in the various stages of the experiment is shown in table 1. Samples were withdrawn after at least four hydraulic displacements when chemical analysis showed steady-state conditions had been attained. Gas samples were periodically taken for analysis. A bladder filled with argon was used to equalise the pressure as samples were withdrawn. This prevented air from being drawn into the system. Gas production was measured by a gas flow meter (Scientific and Projections, Ltd, Footscray, Kent, England).

(b) Chemical analysis

General chemical analysis was performed on an AutoAnalyser (Technicon, Tarry Town, New York). C.O.D. was determined by the method of Adelman (1968), which involves measurement of the colour change from hexavalent to trivalent chromium. Digestion is continuous, using a high temperature heating bath in an entirely closed system.

Ammonia and total nitrogen were measured colorimetrically following the phenol-hypochlorite reaction using a nitroprusside catalyst (Harwood and Huyser, 1970).

Total and orthophosphate were determined spectrophotometrically, using an acidified solution of ammonium molybdate, containing ascorbic acid and a small amount of antimony (Murphy and Riley, 1962).

Nitrate was measured by the sodium salicylate method described by Müller and Widemann (1955), while nitrite was determined by diazotisation, measuring diazonium colorimetrically after combination with sulphanilic acid (Montgomery and Dymock, 1961). No reliable reproducible method could be found for

TABLE 1 : Composition of feed to denitrifying unit  
in the different stages of the experiment

Stage	NO <sub>3</sub> -N (mgℓ <sup>-1</sup> in final mixture)	Methanol (mgℓ <sup>-1</sup> in final mixture)	Supernatant of settled sewage (part of final mixture)
1	300	1200	50%
2	150	600	50%
3	75	300	50%
4	37,5	150	50%
5	37,5	0	50%

measuring methanol concentrations by chemical analysis. This was not an insurmountable difficulty, however, as Whittenbury et al. (1970) found that methanol is transformed into no compounds other than cell constituents and CO<sub>2</sub> by denitrifying bacteria. C.O.D. was used as a measure of dissolved organic carbon compounds in the denitrifying unit.

(c) Gas analysis

Gas samples were routinely analysed on an F and M gas-chromatograph on a Poropak S (100-200 mesh) column with argon as carrier gas. For separation of nitrogen and oxygen, samples were analysed on a molecular sieve column.

(ii) Formulation of a suitable isolation medium for denitrifying bacteria

The medium was formulated around a phosphate buffering system, as Chamroux (1966) found that, in marine sediments, denitrification was better at low C:P ratios.

Ferrous sulphate was added, as spectrographic analysis of nitrate reductase has shown the presence of about forty molecules of bound iron per protein molecule (Taniguchi and Itagaki, 1959). Iron is also essential for a wide range of other enzymes, particularly cytochromes. At least half of the iron found in denitrifying bacteria is present as cytochrome C, which has been shown to function in the electron transfer sequence (Nicholas, 1963). Sulphate would provide the sulphur essential for protein synthesis.

Hydroxylamine is considered to be an intermediate in the reduction of nitrate (Iwasaki and Mori, 1958). Both assimilatory and respiratory hydroxylamine reductase require flavin and manganese for maximum activity (Kono et al., 1957; Nicholas, 1963). Manganese was added in the form of manganese chloride.

Magnesium sulphate was provided, as magnesium stimulates the activity of the thiaminoprotein enzymes during CO<sub>2</sub> production. It has been routinely used in culture of both denitrifying bacteria (Davies and Toerien, 1971) and methane oxidising bacteria (Silverman, 1964).

Although molybdenum does not appear to have been used for the growth of methane oxidising bacteria in the past (Silverman, 1964), previous studies have involved aerobic bacteria with no provision made for the requirements of denitrification. Molybdenum has been established as a functional constituent of nitrate reductase (Nicholas et al., 1954; Nicholas and Nason, 1954a,b), acting as a functional electron carrier after flavin (Nicholas and Stevens, 1956). Because it was intended to isolate methane oxidising bacteria capable of using nitrate respiration, molybdenum was added to the medium in the form of sodium molybdate.

Although some unusual additives have previously been used in the culture of methane oxidising bacteria, such as aged seawater (Hutton, 1948), calcium pantothenate and a concentrated water extract of agar (Dworkin and Foster, 1956), Leadbetter and Foster (1958) found that calcium chloride would serve just as well. This was used in the medium described here. No nitrogen was present in the ammonia form and all nitrogen for cell synthesis had to be obtained by nitrate reduction. All ingredients were dissolved separately in distilled water and then mixed together in the final volume of water, to prevent the formation of insoluble precipitates.

A vitamin solution (Table 2) was added, having been used previously in successful long-term experiments on denitrification. The final medium was essentially similar to that used by Davies et al. (1971), the yeast extract and Bacto-peptone being omitted as they proved to be unnecessary.

TABLE 2 : Composition of the vitamin solution

Vitamin	mg $\ell^{-1}$ distilled water
Thiamine hydrochloride	250
L- ascorbic acid	250
Riboflavin	250
Biotin	1000
Cobione	60
Pyridoxin hydrochloride	500
Folic acid	100
Para-aminobenzoic acid	500
Nicotinic acid	250
Choline	250
$\alpha$ -tocopherol	250

The final basic denitrifying medium (Table 3) was used in all experiments, with the addition of various organic carbon compounds and different concentrations of potassium nitrate.

TABLE 3 : Composition of basic denitrifying medium

Constituent	Quantity per litre distilled water
$K_2HPO_4$	3,0 g
$KH_2PO_4$	2,25 g
$MgSO_4 \cdot 7H_2O$	200 mg
$NaMoO_4 \cdot 2H_2O$	150 mg
$CaCl_2 \cdot 2H_2O$	40 mg
$FeSO_4 \cdot 7H_2O$	5,5 mg
$MnCl_2 \cdot 4H_2O$	1,0 mg
Vitamin solution	1,0 ml

The medium was sterilised by filtering through a 0,2  $\mu$  membrane and tested for sterility by the plating of a sample before it was used as a feed for the denitrifying unit.

(iii) Isolation and identification of numerically important denitrifying bacteria

Samples were withdrawn from the denitrifying unit at regular intervals. Microscopic examination revealed that the population was extremely heterogeneous, containing straight and curved rods, cocci and spirilla, as well as ciliate and flagellate protozoa. Many of these organisms were latent members of the population, being introduced with the settled sewage. In order to determine the number of the bacteria actively denitrifying the Most Probable Number (MPN) technique was used (Standard Methods, 1965).

This involved twelve tenfold dilutions of an original sample of 1 ml taken from the suspension in the unit. From each dilution level 1 ml of suspension was transferred by hypodermic syringe into five replicate stoppered test tubes containing 9 ml of nitrate broth (Table 3) and a Durham tube under argon atmosphere. A similar sample was diluted after heating to 80°C for ten minutes, in order to preserve only sporeformers in the population.

Loopfulls of suspension were taken from the tubes of the highest dilution showing positive nitrogen production, streaked on a nitrate agar (Table 4) and incubated aerobically at 30°C. The resulting colonies were picked off and re-streaked twice to purify. Isolates were maintained on nitrate agar slopes and identified to generic level according to Skerman (1959).

TABLE 4 : Composition of nitrate agar

Constituents	g l <sup>-1</sup>	
KNO <sub>3</sub>	2,0	A
'Lab-lemco' meat extract (Oxoid)	0,5	
Bacto-peptone (Difco)	1,0	
Malic Acid (Technical)	2,0	
Bacto-agar (Difco)	15,0	B

Part A was dissolved in distilled water and made up to 1 litre. The pH was adjusted to 7,2 with 1N NaOH. The agar (B) was added and the mixture boiled to dissolve. The resulting solution was decanted into tubes in 9 ml aliquots and sterilized at 121°C for 15 min.



(iv) Specificity of isolates to methanol as a hydrogen donor

To test the specificity of isolates to methanol as a carbon source the isolates, presumed sporeformers and presumed nonsporeformers, were inoculated separately into nitrate broth containing  $3 \text{ g l}^{-1} \text{ KNO}_3$  and  $10 \text{ g l}^{-1}$  of a carbon source under an argon atmosphere. The four hydrogen donors selected were casamino acids (Difco), sodium malate, sodium lactate and methanol. Gas produced was analysed on an F and M gaschromatograph, using a Poropak column.

2. ISOLATION OF BACTERIA CAPABLE OF USING METHANE FOR DENITRIFICATION

(i) Enrichment

A 10 ml sample of suspension taken from the suspended growth unit receiving methanol as a carbon source was inoculated into a 2l aspirator, containing 1 litre of basic denitrifying medium plus  $1 \text{ g l}^{-1}$  of nitrate nitrogen but no organic carbon. Methane was passed through a sterile cottonwool filter and bubbled continuously through the culture fluid. A similar sample was inoculated into a medium designed for the culture of Methanomonas (Table 5), through which methane was similarly bubbled.

(ii) Isolations

After several days incubation at  $30^\circ\text{C}$  a fairly thick suspension had developed in the denitrifying medium, but no growth occurred in the Methanomonas medium. A sample was taken from the denitrifying suspension and passed through a series of tenfold dilutions, using stoppered test tubes containing 9 ml dilution fluid under a methane atmosphere (99.97%, ultra high purity, Matheson gas products) by means of sterile 1 ml syringes (Toerien and Siebert, 1967). From the  $10^{-5}$  to  $10^{-8}$  dilutions aliquots were dispensed into each of four replicate Astell roll tubes kept molten at  $50^\circ\text{C}$ . The resulting

TABLE 5 : Composition of Methanomonas growth medium  
(adapted from Skerman, 1959)

Constituent	Quantity per litre distilled water
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1,0 mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	200 mg
$\text{H}_3\text{BO}_3$	60 mg
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	38 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	300 mg
$\text{NaMoO}_4 \cdot 14\text{H}_2\text{O}$	150 mg
KCl	40 mg
$\text{CaCl}_2$	15 mg
$\text{KNO}_3$	2,38 mg
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	200 mg
$\text{Na}_2\text{HPO}_4$	210 mg
$\text{NaH}_2\text{PO}_4$	90 mg
$\text{NaHCO}_3$	3,73 g

mixture was cooled as the roll tubes were spun, resulting in a thin layer of solidified agar adhering to the walls of the tubes. The tubes were then incubated at 30°C. Colonies forming on the agar layer were picked off and streaked to purify in similar roll tubes.

Other test tubes were prepared, each containing a Durham tube and 10 ml carbon free denitrifying medium (Table 3 with 1 g  $\text{KNO}_3 \text{ l}^{-1}$ ) under methane. These tubes were inoculated with the isolates obtained previously, in order to verify which isolates were able to denitrify with methane as the sole carbon source. When gas appeared in the Durham tube it was analysed on an F and M gaschromatograph with a Poropak column. Isolates found to produce nitrogen were subjected to diagnostic tests and identified according to Skerman (1959).

(iii) Specificity of isolates to methane

Isolates were inoculated into stoppered test tubes containing Durham tubes, an argon atmosphere and denitrifying medium containing  $1 \text{ g N } \ell^{-1}$  and one of the following compounds at a concentration of  $4 \text{ g C } \ell^{-1}$ ; methanol, ethanol, malate, lactate. Another series of tubes, containing Durham tubes, 9 ml basic denitrifying medium without any carbon sources and an atmosphere of 70%  $\text{H}_2$  and 30%  $\text{CO}_2$ , was inoculated, in order to discover if any of the isolates could use hydrogen directly in the process of denitrification.

A stock culture of Micrococcus denitrificans Beijerinck (American Type Culture Collection No. 13543) was also subjected to all the above tests. One of the isolates (Alcaligenes sp.) was inoculated into four 3 litre culture vessels, each containing 1 litre of basic denitrifying medium,  $1 \text{ g } \ell^{-1}$  nitrate nitrogen and  $2.5 \text{ g C } \ell^{-1}$  of methanol, ethanol, lactate or malate. Suspended solids determinations from these cultures were made daily, to determine comparative growth rates with the four different carbon sources. These determinations involved centrifugation of 10 ml aliquots of bacterial suspension for 30 min. at 80 000 xg. The supernatant was poured off and the precipitate dried at  $105^\circ\text{C}$  for 24 hours before weighing.

Bacteria previously isolated from the methanol suspended growth units were inoculated into sealed test tubes with methane as the sole carbon source, to see if these isolates could denitrify under such reputedly specialised conditions.

3. THE EFFECT OF pH AND TEMPERATURE ON BACTERIA  
ABLE TO USE METHANE FOR DENITRIFICATION

(i) The design of a chemostat for the study of  
pure cultures of denitrifying bacteria

(a) Culture vessel

It was intended to use completely synthetic growth media in which every constituent was known and controlled. For this purpose the unit could not be too large, otherwise medium preparation would be too costly and time consuming. However Dawson (1963) and Herbert et al. (1965) indicated that the volume of sample drawn off should never be more than five per cent of the working volume if steady-state conditions are not to be disrupted. In order to allow sufficient sample to be withdrawn for satisfactory analysis, the working volume was established at one litre. The cyclone column (Fig 2) was constructed with a diameter of 70 mm and a total length (excluding the cap) of 680 mm. With all three ports to the volume control limb open, this brought the total working volume to one litre, including the fluid in the recirculation limb. This volume can be easily adjusted by closing off two of the connections to the volume control limb, leaving the other open. When the chemostat is in action, the culture volume will stabilise at one litre, 800 ml or 600 ml, depending on which limb is left open.

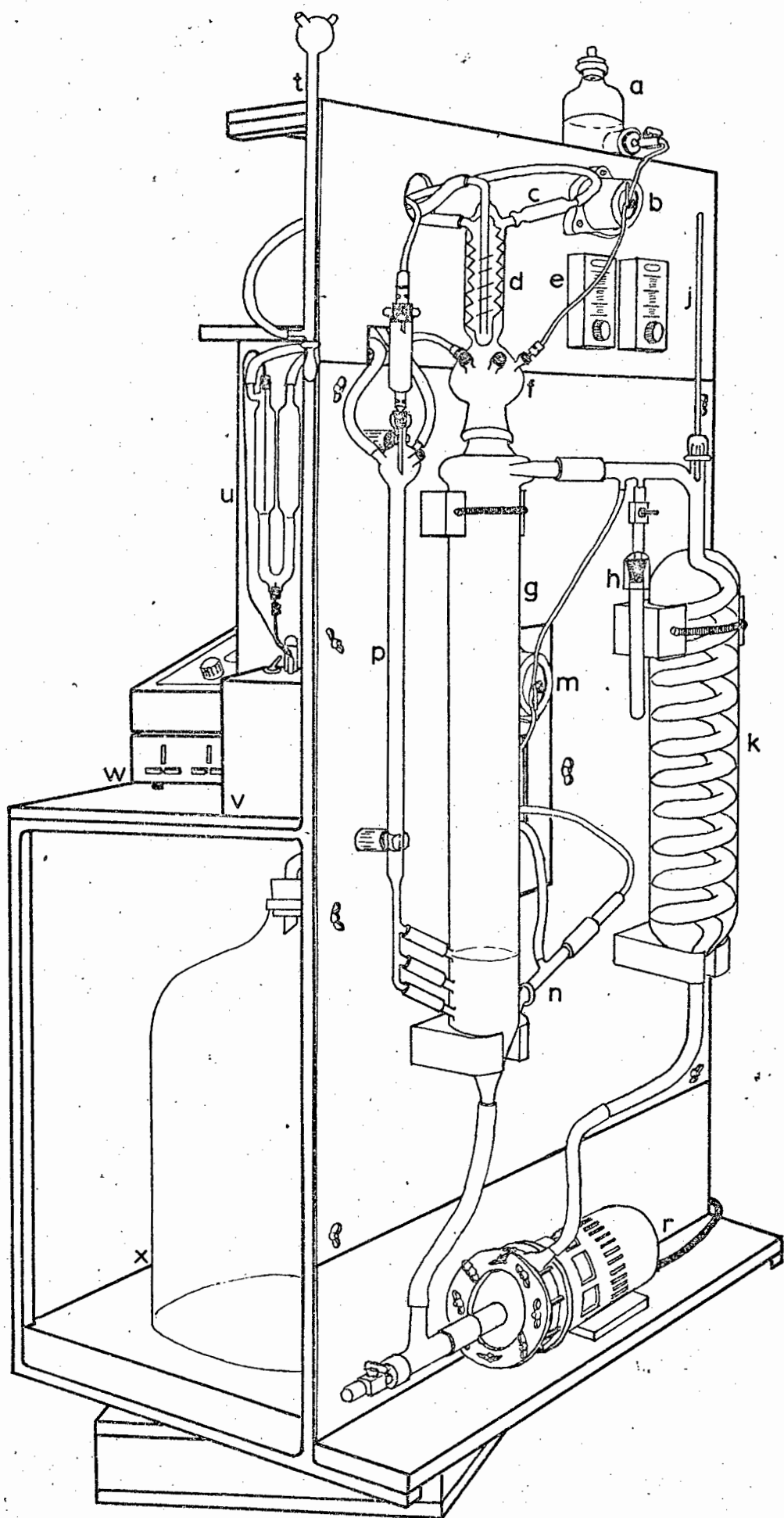
The inlet tube to the cyclone column was tapered, the internal diameter decreasing from 11 mm to 6 mm, in order to ensure a vigorous spiral action when the fluid spins down into the reservoir, preventing growth from occurring above the surface of the main body of the culture fluid.



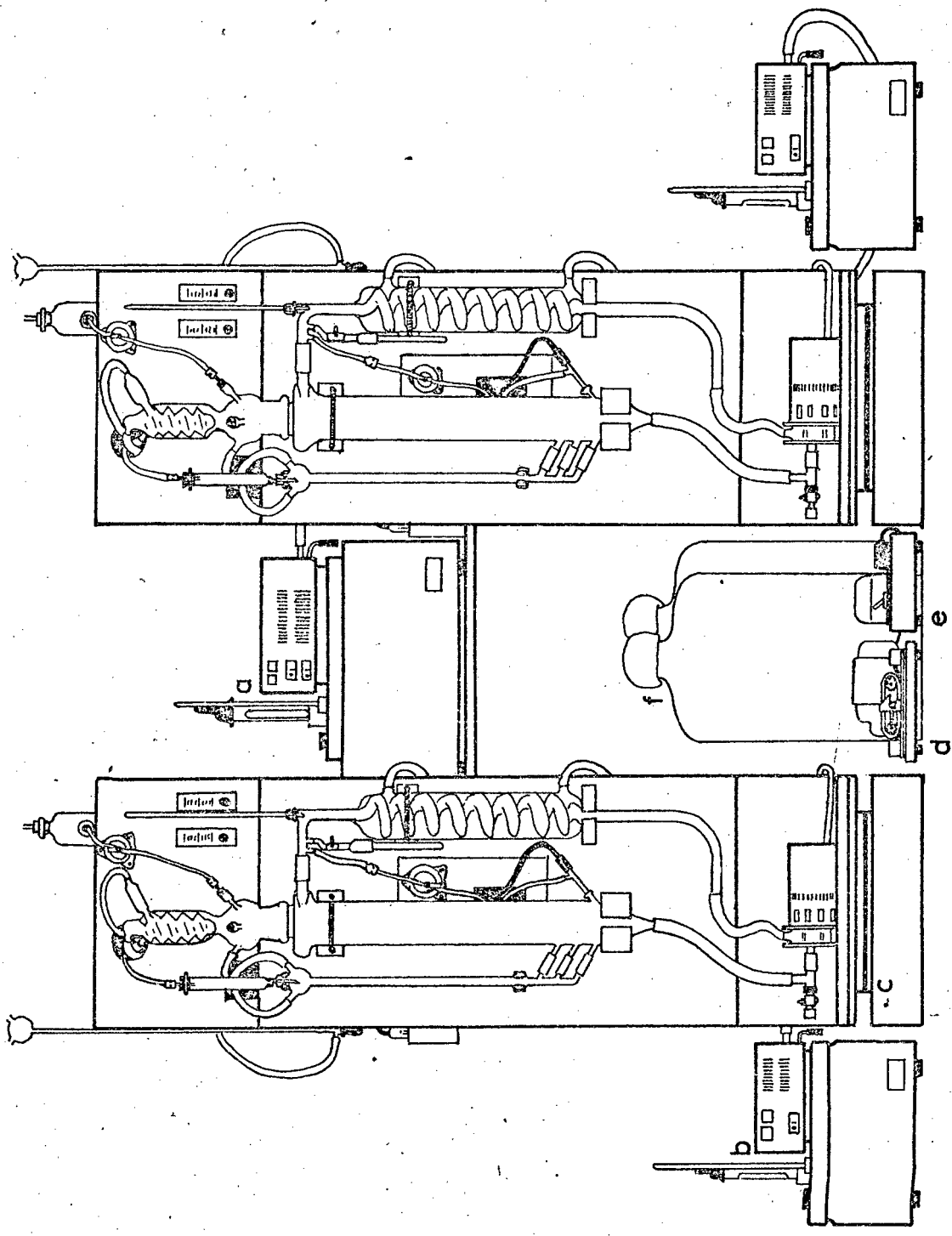


FIGURE 2 : Continuous culture apparatus designed to study denitrification

- Key
- a acid/alkali reservoir
  - b pH control magnetic pinch-valve
  - c gas effluent filter
  - d gas effluent condenser
  - e gas flow control valves
  - f cyclone column cap with the following ports:
    - i alkali addition port
    - ii acid addition port
    - iii connection to volume control manometer
    - iv inoculation port
  - g cyclone column
  - h sample withdrawal point
  - j thermometer
  - k temperature control spiral in recirculation limb
  - m volume control magnetic pinch valve
  - n combined glass-calomel pH electrode
  - p volume control limb, with the following ports:
    - i medium inlet cannula
    - ii connection to volume control manometer
    - iii, iv two gas inlet ports
  - r magnetic drive circulation pump
  - t gas flow meter
  - u volume control manometer
  - v volume control relay
  - w pH meter - titrator
  - x culture harvest vessel







Connections were made by seamless latex rubber tubing, which enabled the chemostat to be autoclaved. This tubing will withstand several sterilisations, but it was changed before each sterilisation in order to avoid splitting or leakage in the middle of an experimental run.

(b) Circulation pump

Circulation of the fluid presented something of a problem. Pumps using a drive shaft are susceptible to infection through the gland of the drive shaft and present difficulties in initial sterilisation of the unit. Pumps with drive heads of metals such as copper were avoided because of possible toxicity to the cells. Magnetic drive pumps proved to be a satisfactory answer to sterilisation and infection problems. Initially stainless steel pump heads were used, but these tended to frequently lose traction and the heavy steel rotors had to be accelerated gradually if the magnetic traction was to be renewed. Eventually magnetic drive pumps with heads of autoclavable polypropylene became available (Model MDX3, March Manufacturing Company, Glenview, Ill., USA). These were found to run reliably for continuous periods of more than a month without ever losing traction. They were modified by the addition of aluminium clamps, so that the polypropylene head could be removed intact from the pump and autoclaved attached to the rest of the denitrifying unit.











(c) Medium supply

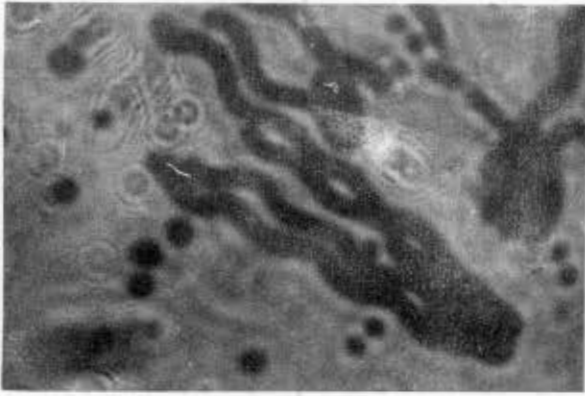
The growth medium was completely synthetic (Table 3). It was contained in 20 litre aspirators which had been autoclaved empty. In order to prevent changes taking place in the medium due to the heat of autoclaving, the medium was sterilised by



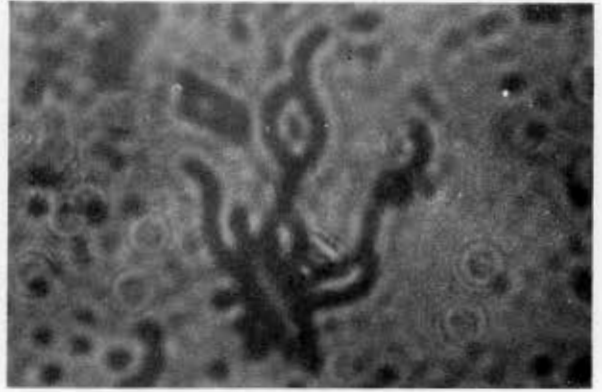
FIGURE 5 : Growth of *Alcaligenes* sp. on various organic compounds

Legend for figure 5

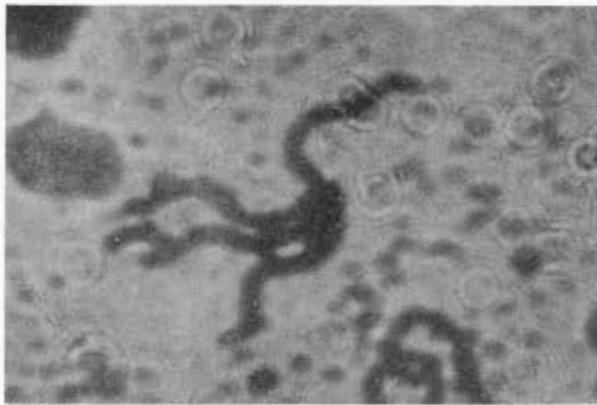
methane	 — 
methanol	 — 
ethanol	 — 
lactate	 — 
malate	 — 



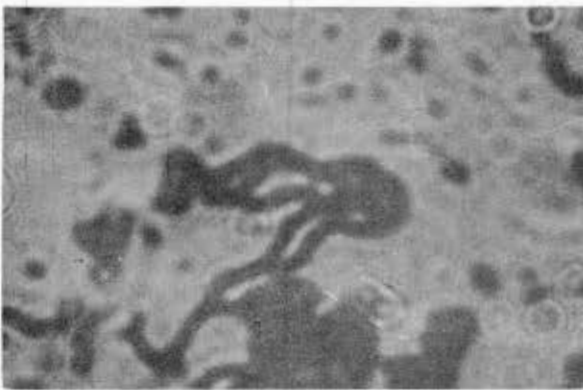
1.



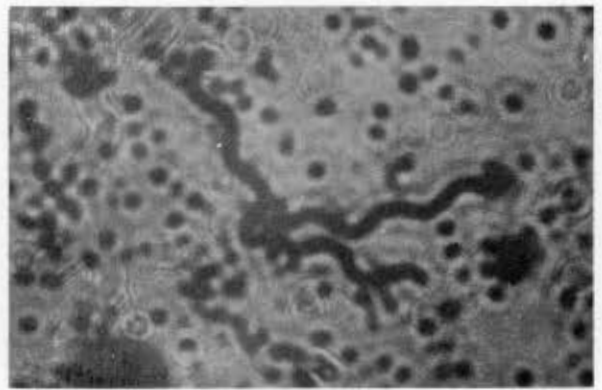
2.



3.



4.



5.

membrane filtration.

The membrane filter unit (Sartorius Membrane Filter GmbH, Göttingen, West Germany) was sterilised and connected aseptically to a needle inserted into the bottom aperture of the aspirator. The fluid was then passed by means of nitrogen gas pressure of  $0,6 \text{ kg cm}^{-2}$  through a 130 mm fibre-glass pre-filter and a 142 mm membrane filter with a pore size of  $0,2 \mu$ . The bottle of medium was then kept at room temperature for several days and checked for sterility before connection to the denitrifying unit.

Medium was fed into the unit by means of a roller type peristaltic pump, operated by a 28 rpm Fracmo motor (Fractional H.P. Motors Ltd., Enfield, England). Pumping rate was controlled by means of different diameters of PVC tubing in the peristaltic pump and a timing device. Medium had to drip down the volume control limb and an additional drip space above this, which effectively prevented any growth back along the feed line.

(d) Volume control

A slow stream of gas ( $\pm 10 \text{ ml min}^{-1}$ ) was passed through the unit via one of the ports in the head of the volume control limb. When the level of the medium in the cyclone column rose enough to cover the inlet from the volume control limb to the cyclone column, a back pressure would be formed in the side limb. The head of this limb also had a connection to a manometer, mounted on a side panel at the left of the unit. This contained a dilute KCl solution, which under pressure made contact between two platinum electrodes connected to a volume control relay, constructed by Electronic Instrumentation, TSD, CSIR, Pretoria. The making of this contact activated a magnetic

pinch valve (Radiometer MNVI, Copenhagen, Denmark), which released fluid from the top of the recirculation limb. When the fluid volume was sufficiently decreased, the back pressure in the volume control limb disappeared, breaking the electrical contact in the manometer and allowing the pinch valve to close again.

(e) Gas supply

Although the volume control system was dependent upon a constant flow of gas through the unit, this had to be kept to a minimum so that gases produced during denitrification could be detected in measurable amounts. Normally argon was used, as this was the carrier gas being used in the gaschromatograph for the detection of  $N_2$ ,  $CO_2$ ,  $H_2$  and  $N_2O$ . Provision was, however, made for mixing gases in the system, as the gas supply facility was duplicated.

The gas flow rate was controlled by a gas flow meter (W. Dwyer Manufacturing Co., Michigan City, Ind., USA), which was normally adjusted to allow a flow rate of about  $10 \text{ ml min}^{-1}$ . Gas passed from here via a sterile cottonwool filter to the head of the volume control limb, which was connected by another filter to the volume control manometer and then via a further filter to the multi-ported cyclone column cap. The volume control limb was connected to the cyclone column by three ports, but only one of the three was actually operational in any experiment, depending on the volume of culture required. Gas passed up the cyclone column, the walls of which were thinly covered by a large area of spiralling culture medium, allowing for adequate gas solution or evolution. The gas then left the cyclone column, after passing over a condenser, which

prevented the exhaust filter from becoming moist and a potential source of infection. From the exhaust filter the gas was piped to a flow meter where the time taken for the evolution of 10 ml of gas could be measured.

(f) Temperature control

The denitrifying unit required water at two different temperatures. The condenser on the cyclone column cap needed cold water at a maximum temperature of 10°C, while the water jacket on the temperature control spiral of the recirculation limb had to be supplied with water at a temperature suitable for experimental conditions. The first requirement was filled by means of a low temperature reservoir (Model K2RD, MGW Lauda, West Germany), which was used to supply water to the condensers of both units. It also fed cold water through the cooling coils of smaller waterbaths (Model K2, MGW Lauda), each of which provided water to the temperature regulating jacket of its respective unit at the desired temperature.

At the top of the recirculation limb a 150°C thermometer was inserted directly into the culture fluid, in order to monitor the exact temperature of the medium. The port for this thermometer was provided with a glass thread, onto which a heat resistant plastic cap (type SQL3, Quickfit and Quartz, Stone, U.K.), was fitted, its enclosed rubber sealing ring preventing seepage. The thermometer was autoclaved in position. This system provided a temperature accuracy of better than 0.1°C, complying with the recommendation of Farrell and Rose (1967) that temperature should not be allowed to vary by more than 0.2°C in meaningful temperature response experiments.



(g) pH control

A combined glass/calomel electrode (No GK 2641C, Radiometer, Copenhagen), was inserted directly into the main body of the culture fluid in the lower part of the cyclone column. This electrode was constructed with a ground glass cone which fitted into a B10 socket on the cyclone column. Experience showed that it was necessary to round the upper shoulder of this socket, otherwise bubbles collected in the small side chamber, removing the electrode from contact with the culture fluid. The electrode had a side arm intended for maintaining the level of KCl. This was connected by latex tubing to a reservoir containing saturated KCl, which was located on the rear shelf next to the pH meter. The KCl therefore exerted a positive pressure on the porous glass membrane of the electrode, preventing seepage of culture fluid into the electrode and ensuring that the porous bulb was not blocked by bacterial growth.

The electrode was connected to a pH meter (Type PMM 28b, Radiometer), which was coupled to a titrator (Type TTT 11b, Radiometer), mounted on a shelf behind the bakelite panel. This unit controlled a magnetic pinch valve (Type MNV1, Radiometer), which regulated the addition of acid or alkali to the culture. The incorporation of a second titrator and valve would make possible two-way control of the pH. This system operated at an accuracy of better than 0.1 pH units.

(h) Layout

(see Fig 3). The chemostats were mounted on a bench top against a wall. In order to facilitate access to the back of the assemblies each unit was erected on a turntable of 40 cm diameter, to allow rotation through 90°.

The frame of each unit was constructed from 20 mm square tubular steel and the shelves from 20 mm thick marine plywood. The front panels were made from 7 mm bakelite, which is almost completely resistant to warping during sterilisation. The upper and lower bakelite panels were permanent fixtures, but the central panel, bearing the glassware, could be removed as a single entity and placed in the autoclave with the chemostat totally assembled.

The glassware was mounted on aluminium blocks and 19 mm bakelite rods and the central panel was secured to the frame by seven brass wing nuts for easy removal. When the panel was removed for sterilisation, a smaller panel measuring 36 cm x 7,5 cm remained, bearing the volume control magnetic pinch valve and a storage clip for the pH electrode during sterilisation.

At the rear of the unit, the pH meter and titrator were on the upper shelf, while the harvest bottle was placed on the lower shelf. The bottles of medium, together with the medium pump and timer, stood on the bench between the two units. The temperature control water baths were on the bench top each at the outer side of its respective chemostat, while the low temperature water bath was on a separate shelf between the units.

(j) Sterilisation

Gas connections were detached beyond the cottonwool filters and Luerlock fittings, such as stopcocks, were covered with aluminium foil. The centre panel was then removed with its glassware and sterilised, together with the medium supply bottle, at  $1,76 \text{ kg cm}^{-2}$  and  $165^{\circ}\text{C}$  for one hour in an autoclave with a drying cycle.

The pH electrode was sterilised by immersion overnight in 0,1 N HCl which had previously been tested and found to be satisfactory. The growth medium was sterilised by membrane filtration and then tested for sterility. This medium was pumped into the chemostat, after the sterile pH electrode had been put into place. The unit was then run for two days prior to inoculation and the medium again tested for sterility. After this test the chemostat was regarded as ready for inoculation.

(ii) Operation of a chemostat containing denitrifying bacteria with the ability to oxidise methane

In view of the claimed specificity of methane oxidising bacteria to single carbon donors, an isolate taken from the methane fed denitrifying unit was grown on three different carbon sources, methane, methanol, and lactate. To the basic denitrifying medium nitrate was added at a concentration of  $1,0 \text{ gN } \ell^{-1}$ , together with methanol or lactate at  $2,5 \text{ g C } \ell^{-1}$ . The methane culture medium contained no dissolved organic carbon, but methane gas was passed through the chemostat in place of argon, allowing methane to pass into solution from the gas phase, during the course of the experiments.

In one series of experiments the temperature was maintained at  $30^{\circ}\text{C}$ , while the pH was either raised or lowered from pH 7,0 in stages of 0,5 pH units, until growth had become sparse and denitrification had virtually ceased. The experiment was repeated using each of the three carbon sources.

In a further series of experiments the pH remained unaltered at 7,0 while the temperature was raised or lowered from  $30^{\circ}\text{C}$  in stages of  $5^{\circ}\text{C}$ . Once again each isolate was tested using each

carbon source.

The growth medium was inoculated with an actively growing, denitrifying suspension of the isolate and maintained without feed until a thick suspension had formed in the cyclone column. Feeding was then begun, allowing a hydraulic residence time of 10 hours. As it is customary to allow three hydraulic displacements to elapse before steady-state conditions can be expected, gas samples were withdrawn from the unit every 40 hours. These were analysed on an F and M gaschromatograph using a Poropak column and argon as carrier gas. Liquid samples of 50 ml were then taken from the culture suspension, after which the chemostat was adjusted to the following stage of the experiment.

Initially the cells in suspension were counted on a Coulter counter (model B with model J plotter). The 2% NaCl solution used as electrolyte was filtered through a 0,2  $\mu$  membrane before use, but this did not completely prevent clogging of the 30  $\mu$  aperture. This method of estimating biomass, which would have yielded information about cell size distributions as well as numbers, if it had been successful, was finally abandoned. Results obtained were unreliable, probably because the aperture was too large to count particles as small as bacteria. Amplification of the signal simultaneously increased the masking caused by electronic noise and did nothing to improve counting reliability.

Biomass was subsequently estimated by measuring the total suspended solids. 10 ml samples of suspension were centrifuged at 75 000 x g for 30 min. The precipitate was then dried for weighing, while the supernatant was used for chemical analyses. Most analyses were performed on an AutoAnalyzer (Technicon, Tarry Town, New York), by the methods described earlier (III, 1, (i)(a)).

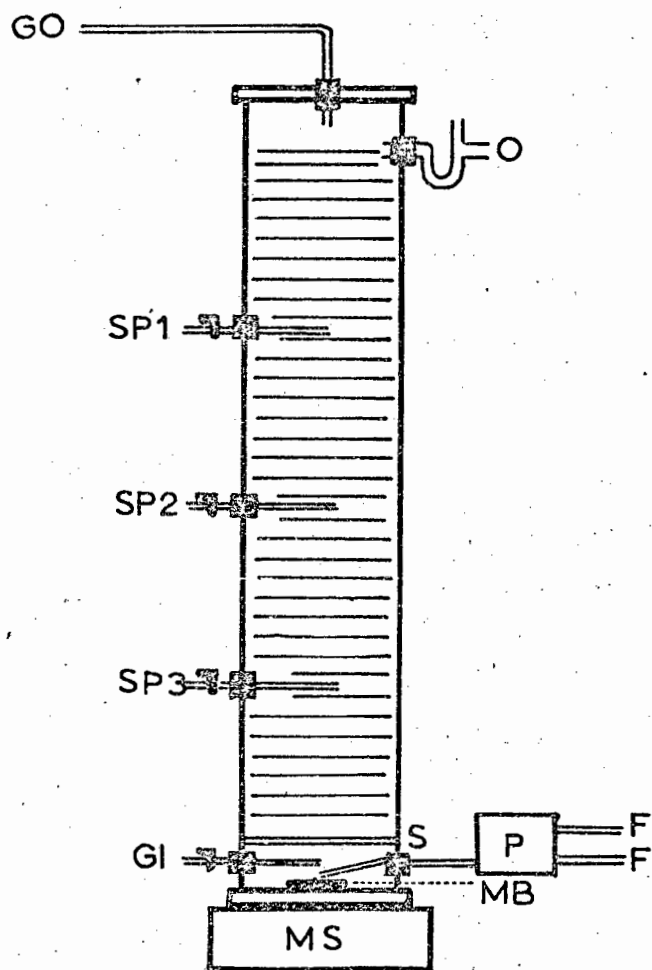
4. OPERATION OF A COMMERCIAL-TYPE DENITRIFYING UNIT  
WITH METHANE AS THE CARBON SOURCE

In order to determine whether results obtained with pure culture could be applied to commercial systems, a laboratory denitrifying unit was used, containing a population derived from settled sewage. The unit was of the packed column type, filled with crushed stones of approximately 2 - 3 cm diameter. This gave the unit a void volume of 5,8 litres (Fig 4). The unit had a mixing space where the feed and gas were mixed by a magnetic stirrer before passing through the packing of the column. The column was operated in a constant temperature room at 30°C. The unit had previously received the supernatant from settled sewage, methanol and a nitrate solution as feed. It was then filled with a denitrifying medium without any dissolved carbon and methane gas was bubbled through the column for three days to allow the population time to adapt to the new feed.

After this adaptation period the unit was fed with a nitrified effluent from a revolving disc type nitrifying unit (Pretorius, 1971) at a rate which gave a hydraulic residence time of 20 hours and supplied nitrate at a rate of 12,9 mg  $\text{NO}_3\text{-N h}^{-1}$ . Effluent gas from an anaerobic digester, with a composition of 60% methane and 40%  $\text{CO}_2$ , was introduced at the bottom of the column at a rate of 11 litres per day, and allowed to bubble through the packing material. The rates at which gas entered and left the unit were measured and analysed for calculation of the rate of denitrification.

Chemical analyses were performed as described earlier.





#### IV. EXPERIMENTS AND RESULTS

The aim of this investigation was, initially, to show that single carbon hydrogen donors could be used efficiently in denitrification. The first step involved demonstrating that methanol was more suitable than the supernatant fluid of settled sewage for achieving denitrification. The bacteria responsible for denitrifying with methanol could then be isolated, identified and tested for their specificity to single carbon hydrogen donors.

This methanol culture was then used as inoculum for a unit receiving methane as the sole carbon source for denitrification. From this enrichment bacteria could be once again isolated and identified. These isolates could then be subjected to continuous pure culture study of the effect of pH and temperature on their growth and denitrifying ability.

##### 1. ISOLATION OF BACTERIA USING METHANOL AS A DONOR FOR DENITRIFICATION (cf. Ch. III, 1).

###### (i) Operation of a denitrifying unit receiving methanol as extraneous carbon source

Initial results from this unit were very disappointing, in that they gave very low denitrification rates. Chemical analysis showed, however, that the population was still adjusting and steady-state conditions were not achieved until six hydraulic residence periods had elapsed. Once steady-state conditions were established, the population adapted normally to experimental changes and samples could be withdrawn four hydraulic displacements after a new feed had been supplied to the unit. As the feed was largely composed of the supernatant fluid from settled sewage, the composition of the feed varied from one stage of the experiment to the next. Each stage, however, was conducted with the same batch of feed, so that



the composition of the feed did not change during any single stage. The 4:1 ratio of C:N recommended by Barth et al. (1968) was used throughout the experiment.

(a) Chemical analysis

The results of these analyses are presented in Table 6.

Initially 97,6% nitrate and nitrite removal and 85,2% total nitrogen removal were obtained. In each experiment a figure of better than 90% denitrification was consistently obtained as long as methanol was added to the feed. When methanol was withheld, this figure fell to only 34%.

Total nitrogen removal was better at higher carbon-nitrate concentrations due to two factors. Firstly the higher nitrate concentrations provided a relatively larger proportion of dissolved nitrogen compounds in the oxidised form, and these could be removed by denitrification. Secondly, at these higher nitrate values large numbers of bacterial cells were produced, as shown by the high suspended solids values. During this growth some of the dissolved ammonia would be assimilated, resulting in a reduction in ammonia nitrogen by the unit.

However, high concentrations of ammonia (9,7 - 22,8 mg  $l^{-1}$ ) passed right through the unit at all stages, and these concentrations remained virtually unchanged at the lower nitrate-methanol concentrations, as little ammonia assimilation took place due to the reduced cell growth.

C.O.D. removal was not very efficient, falling in the range of 38,6% - 65,8% in the experiments involving methanol addition. This appears to be due to excess methanol in the feed, which indicates that the 4:1 ratio of methanol to  $NO_3-N$

TABLE 6 : Results of operation and chemical analysis of suspended growth unit with methanol as extra source of carbon

	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	Feed	Overflow	Feed	Overflow	Feed	Overflow	Feed	Overflow	Feed	Overflow
pH range		9,00-9,30		8,80-9,00		8,40-8,60		8,00-8,20		7,60-7,80
Suspended solids (mg $\ell^{-1}$ )		479,0		244,0		178,0		82,0		48,0
COD (mg $\ell^{-1}$ )	1980,0	679,0	1550,0	700,0	920,0	620,0	880,0	540,0	360,0	41,0
Methanol (mg $\ell^{-1}$ )	1200,0	N.D.	600,0	N.D.	300,0	N.D.	150,0	N.D.		
Org-P (mg P $\ell^{-1}$ )	0,4	0,5	0,7	0,6	1,3	0,8	0,4	0,3		
Ortho-P (mg P $\ell^{-1}$ )	3,8	2,3	4,4	2,0	2,9	1,3	4,0	4,0	2,5	2,6
Org-N (total) (mg N $\ell^{-1}$ )	14,9	29,5	8,7	17,9	12,1	14,6	16,1	16,2	18,2	14,2
Org-N (in solution) (mg N $\ell^{-1}$ )		7,1		8,2		7,8		8,4		9,2
NH <sub>3</sub> -N (mg N $\ell^{-1}$ )	14,4	9,7	21,0	10,8	24,3	11,0	27,8	26,6	22,8	22,8
NO <sub>3</sub> -N (mg N $\ell^{-1}$ )	300,0	4,0	150,0	0	75,0	0	37,5	0	37,5	23,5
NO <sub>2</sub> -N (mg N $\ell^{-1}$ )	5,0	2,8	2,0	2,1	0	1,6	0	0	0	0
N <sub>2</sub> -gas (mg N pro- duced 4 h <sup>-1</sup> )		292,0		144,0		72,3		32,9		12,75
Total N (all forms) (mg N $\ell^{-1}$ )	343,3	333,0	181,7	174,8	111,4	99,5	81,4	76,0	78,5	73,3
NO <sub>3</sub> -N, NO <sub>2</sub> -N removal		97,6%		97,3%		97,7%		100,0%		37,3%
Denitrification		95,7%		94,8%		96,4%		94,0%		34,0%
Total N removal		85,2%		79,4%		65,0%		40,5%		17,8%
COD removal		65,8%		55,0%		32,6%		38,6%		88,6%

recommended by Barth et al. (1968) is far too high. The discrepancy is probably caused by adherence to anaerobic conditions in the experiment reported here, while Barth et al. were introducing extremely highly oxygenated nitrified effluents into their denitrification tank.

(b) Gas analysis

The unit was flushed with argon before commencement of the experiment. The composition of the gas phase at each stage in the experiment is presented in Table 7.

TABLE 7 : Composition of the atmosphere in a suspended growth unit with methanol as an extraneous carbon source

Stage number	Gas composition (%)				
	1	2	3	4	5
NO <sub>3</sub> -N (mg N ℓ <sup>-1</sup> )	300	150	75	37,5	37,5
Methanol (mg C ℓ <sup>-1</sup> )	1200	600	300	150	0
N <sub>2</sub>	97,80	98,40	98,00	97,10	93,80
N <sub>2</sub> O	0,25	0,08	0	0	0
O <sub>2</sub>	0,41	0,25	0,57	0,38	0,69
CO <sub>2</sub>	0,18	0,23	0,24	0,33	1,45
CH <sub>4</sub>	0,02	0,25	0,52	1,03	2,22
H <sub>2</sub>	0	0	0	0	0
Total	98,66	99,21	99,33	98,84	98,16

Traces of hydrogen were detected in stages 3 - 5

At high nitrate-methanol concentrations N<sub>2</sub>O was formed, but at 75 mg N ℓ<sup>-1</sup> this gas was not detected. Traces of hydrogen appeared in the gas phase once N<sub>2</sub>O production ceased. The small quantities of CO<sub>2</sub> recorded were due to the pH in the unit

always being above 7,4, resulting in the  $\text{CO}_2$  produced remaining in solution.

Methane was detected in quantities from 0,02 - 2,22%. This was due to methane bacteria present in settled sewage. The methane concentration increased proportionately as the rate of nitrogen production diminished. It is difficult to explain the low concentrations of oxygen in the gas phase (0,41 - 0,69%) which could perhaps be ascribed to leakage in the system.

(ii) Isolation and identification of numerically important denitrifying bacteria

(a) Numbers of viable cells present

The numbers of bacteria detected by the two different methods differed considerably. In the second stage, when suspended solids were recorded as  $244 \text{ mg l}^{-1}$  total bacteria numbered  $17 \times 10^6 \text{ ml}^{-1}$ , while there were only  $109 \text{ ml}^{-1}$  presumed sporeformers. In the third stage, with suspended solids at a level of  $17,8 \text{ mg l}^{-1}$ , the total bacteria numbered  $542 \times 10^4 \text{ ml}^{-1}$ , of which only  $41 \text{ ml}^{-1}$  were presumed sporeformers. Sporeformers apparently formed a very small part of the population. Because of the relatively insignificant contribution of presumed sporeformers to the population, no microscopic examination was made to verify that spores were present.

As the experiment progressed microscopic examination showed that Gram negative, flagellate, rod shaped bacteria were becoming dominant in the population.

(b) Identification of isolates

Of a total of twenty eight isolates, twelve proved to be members of the genus Alcaligenes, while six others belonged to

the closely related genus Achromobacter. Seven isolates were strains of Pseudomonas. There were two sporeforming bacteria isolated, both of which were identified as Bacillus strains. The remaining isolate was classified as belonging to the genus Micrococcus.

To test the specificity of isolates to methanol each was inoculated into 1 litre of nitrate broth (Table 3) containing  $3 \text{ g l}^{-1} \text{ KNO}_3$  and  $10 \text{ g l}^{-1}$  of one of sodium malate, casamino acids, sodium lactate or methanol. All isolates grew with each carbon source, showing no specificity at all to single carbon compounds.

(iii) Specificity of isolates to methanol

A non-sporeformer (Alcaligenes) and a sporeformer (Bacillus) have been selected for discussion as typical of their respective groups of isolates.

Strong growth was recorded in each batch culture, especially those of Alcaligenes sp., in which nitrate was completely absent at the termination of the experiment. Production of  $\text{N}_2\text{O}$  by this isolate was very marked when grown on casamino acids, when it comprised over 60% of the gas produced, compared with about 25% of nitrogen gas. Traces of  $\text{N}_2\text{O}$  were produced from the malate and methanol cultures, but none at all from the lactate culture. The casamino acid culture also showed the least  $\text{N}_2$  production, indicating that these electron donors induced nitrate reduction to  $\text{N}_2\text{O}$  rather than  $\text{N}_2$ .

Bacillus sp. showed very low rates of denitrification during which  $\text{N}_2\text{O}$  was never produced. Hydrogen production was detected however, in all except the methanol culture. The greatest hydrogen production was detected in the lactate culture, which also produced

surprisingly large amounts of  $\text{CO}_2$ .

These gas production patterns are interesting, as they agree with the earlier observation that in the denitrifying unit,  $\text{N}_2\text{O}$  and  $\text{H}_2$  production did not seem to occur simultaneously.

It would appear that  $\text{N}_2\text{O}$  production and  $\text{N}_2$  production are due to separate mechanisms. Some bacteria have both of these pathways, each being stimulated by a suitable substrate.

The gaseous nitrogen compound produced as an end product in denitrification is dependent on the nature of the electron donor. Some bacteria possess a mechanism which will produce  $\text{N}_2\text{O}$  as an end product under appropriate conditions, while others produce only  $\text{N}_2$  under the same circumstances.

## 2. ISOLATION OF BACTERIA CAPABLE OF UTILISING METHANE AS A HYDROGEN DONOR IN THE PROCESS OF DENITRIFICATION

Methanol was found to be a very suitable hydrogen donor for denitrification. The bacteria capable of using methanol were by no means specific to single carbon compounds. If methane, a similar compound, could be found to be so effective, it could be the ideal source of hydrogen donors for commercial denitrification plants.

In order to verify this, however, it was necessary to obtain pure cultures of denitrifying bacteria able to use methane. Suitable enrichment media were consequently prepared and inoculated (cf. Ch. III, 2).

### (i) Enrichment

After several days incubation at  $30^\circ\text{C}$  a fairly thick suspension had developed in the nitrate broth receiving methane as the sole source of carbon. Analysis showed that 51% denitrification had taken place in this unit.

The absence of any growth or gas production in the Methanomonas growth medium showed that growth recorded in the denitrifying medium was due to normal denitrifying bacteria making use of methane heterotrophically as a hydrogen donor in nitrate respiration, not bacteria specially adapted for methane utilisation, similar to Methanomonas.

(ii) Isolation and identification

Numerically important methane utilising bacteria were isolated from the denitrifying unit as described in Ch. III, 2, (ii). These isolates were inoculated in pure cultures into a series of sealed tubes containing otherwise carbon free denitrifying medium saturated with methane, under a methane atmosphere.

Strong growth occurred in most of the tubes, gas appearing in the enclosed Durham tubes. Analysis on a gaschromatograph showed that nitrogen gas was being produced.

Eight of the nine isolates showing positive denitrification belonged to the genus Alcaligenes, while the other was identified as a species of Achromobacter, a physiologically similar type. Photographs were taken of these isolates, when they were stained with Leifson's flagellar stain. The photographs can be found in plates 1 and 2. Similar bacteria have been routinely isolated in large numbers from conventional denitrifying systems (Davies and Toerien, 1971). Methane oxidising denitrifying bacteria are essentially the same as bacteria normally found in denitrifying systems, therefore it should be a simple matter to induce an existing denitrifying system with an established denitrifying population to use methane as a hydrogen donor.

Bacteria isolated from the methanol denitrifying unit were inoculated into similar tubes containing carbon free denitrifying medium and a methane atmosphere. Thirteen of these twenty eight isolates grew and produced gas, which was analysed as  $N_2$  and  $CO_2$ . Of these isolates nine proved to be members of the genus Alcaligenes, two others were strains of Pseudomonas, while the remaining two were classified as Achromobacter and Bacillus.

The breakdown of the isolates able to use methane shows that in populations enriched with methanol and those enriched with methane, Alcaligenes is the predominant genus.

The stock culture of Micrococcus denitrificans (ATCC 13543) did not grow anaerobically in the presence of methane and nitrate, despite the ability of strains of this species to use gaseous hydrogen in denitrification (Verhoeven, 1952).

(iii) Specificity to methane

All isolates taken from both denitrifying enrichments which showed the ability to denitrify using methane as the hydrogen donor were capable of using all the other carbon compounds tested. Nitrogen was produced when isolates were provided with methanol, ethanol, malate or lactate as the sole carbon source. No  $N_2O$  production was recorded.

A comparison of the amount of growth on various carbon sources of one strain of Alcaligenes isolated from the methane enrichment is depicted in Fig. 5.

The isolate grew and denitrified on all of the carbon sources supplied. Although the rate of growth was initially fastest with ethanol as the carbon source, growth in the lactate culture eventually



PLATE 1    Photographs of bacteria able to denitrify using  
methane as a hydrogen donor (Leifson's Stain)

Isolate	1	<u>Alcaligenes</u> sp.	x	8000
	2	<u>Alcaligenes</u> sp.	x	6400
	3	<u>Achromobacter</u> sp.	x	6400
	4	<u>Alcaligenes</u> sp.	x	6400
	5	<u>Alcaligenes</u> sp.	x	4000

produced the greatest cell mass.

Growth on methane started very slowly, but after some time this culture produced greater cell mass than the culture grown on methanol. This accords well with the findings of Whittenbury et al. (1970), who reported that methanol eventually yielded 20% less dry weight of cells than did methane.

### 3. THE EFFECT OF pH AND TEMPERATURE ON BACTERIA ABLE TO USE METHANE FOR DENITRIFICATION

Methane oxidising bacteria have been previously thought to be specific to single carbon compounds. This has already been shown to be inapplicable to methane oxidising denitrifying bacteria. A pure culture of methane oxidising denitrifiers was grown in a chemostat in order to determine the effect on growth and denitrifying capacity of different environmental conditions when grown on methane, methanol or lactate (cf. Ch. III, 3).

#### (i) pH

The fluctuations in biomass and denitrifying activity of one Alcaligenes isolate have been presented in Table 8. These variations have also been presented graphically in figures 6 - 8. The bacteria examined showed little sensitivity to pH over a wide range. The extent of this tolerance was dependant on the nature of the carbon source supplied as electron donor. The optimum pH range for denitrification was between 7,0 and 8,5, with a marked decrease in efficiency below pH 6,0 or above 9,0. This accords well with the findings of Karlsen (1938), working with Pseudomonas aeruginosa, who reported notable denitrification over a range of pH 5,8 to 9,2, with optimum activity between pH 7,0 and 8,2. This would make buffering or pH control important, as the pH rises during denitrification, a point illustrated during operation of a denitrification unit on methanol

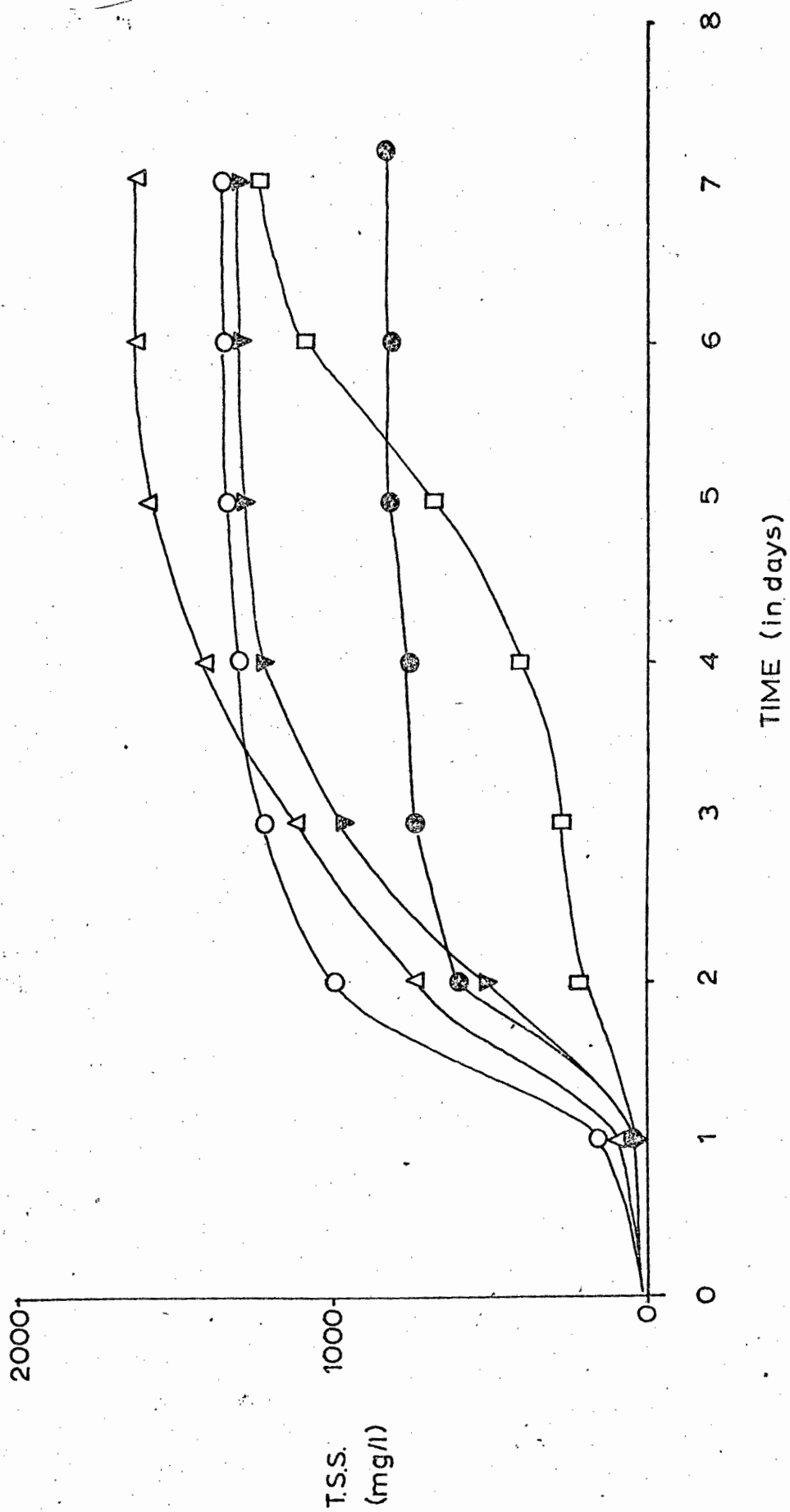


TABLE 8 : Cell production and denitrification by *Alcaligenes* sp. at different pH values with a variety of carbon sources

pH	Methane		Methanol		Lactate	
	TSS (mg l <sup>-1</sup> )	% Denitrification	TSS (mg l <sup>-1</sup> )	% Denitrification	TSS (mg l <sup>-1</sup> )	% Denitrification
3,0	-	-	-	-	84	10,6
3,5	27	-	-	-	130	11,8
4,0	49	1,2	20	-	139	12,7
4,5	100	10,2	120	8,0	255	20,2
5,0	178	19,8	388	56,3	353	23,5
5,5	123	18,9	212	55,7	527	28,2
6,0	197	25,0	480	75,8	535	30,0
6,5	227	32,3	792	86,0	594	43,5
7,0	535	40,5	534	78,1	666	61,1
7,5	830	45,6	391	70,5	695	67,0
8,0	845	43,8	440	70,2	852	69,3
8,5	941	46,8	519	78,6	988	60,4
9,0	765	39,6	421	62,8	796	49,3
9,5	790	41,8	606	64,4	533	41,7
10,0	267	25,7	410	35,8	157	30,2
10,5	344	22,0	278	17,3	149	18,6
11,0	280	16,9	187	8,5	155	9,2
11,5	255	9,8	-	-	51	8,1

(Table 7). A rise much above pH 9,0 would severely limit the efficiency of the system.

The optimum pH range recorded in these experiments agrees fairly well with that proposed by Rabotnowa (1963), who suggested that the fastest generation time of Alcaligenes faecalis could be obtained somewhere in the range pH 6,9 - 9,7.

Wijler and Delwiche (1956) reported that the gases evolved by mixed cultures varied with fluctuations in the pH. They found that at neutral or alkaline pH  $N_2O$  was produced, but once the pH rose above 7,3 the  $N_2O$  was replaced by  $N_2$  as an end product.

Although  $N_2O$  production in small quantities has been recorded in studies on other isolates taken from a normal denitrifying unit receiving lactate as a hydrogen donor, the isolate able to use methane which is currently under consideration, never produced any gasses other than  $N_2$ ,  $CO_2$  and small amounts of  $H_2$ . It is suggested that Wijler and Delwiche in working with a mixed population may have selected different bacteria as they changed the pH. The different nitrogen containing gasses produced would be due to the presence of bacteria normally producing them, rather than to induced changes in the metabolic patterns of bacteria already present.

#### (ii) Temperature

The effects of temperature on biomass and denitrification have been presented in Table 9, while the fluctuations in the suspended solids and denitrification values have been presented graphically in figures 9 - 11.

It was apparent that optimum growth and denitrification rates were achieved at temperatures around  $30^{\circ}C$ , in all except the methane



FIGURE 6 : Percentage denitrification and suspended solids  
at various pH values with methane as the hydrogen donor

Key to figures 6 - 8

——— Percentage denitrification  
----- Total suspended solids

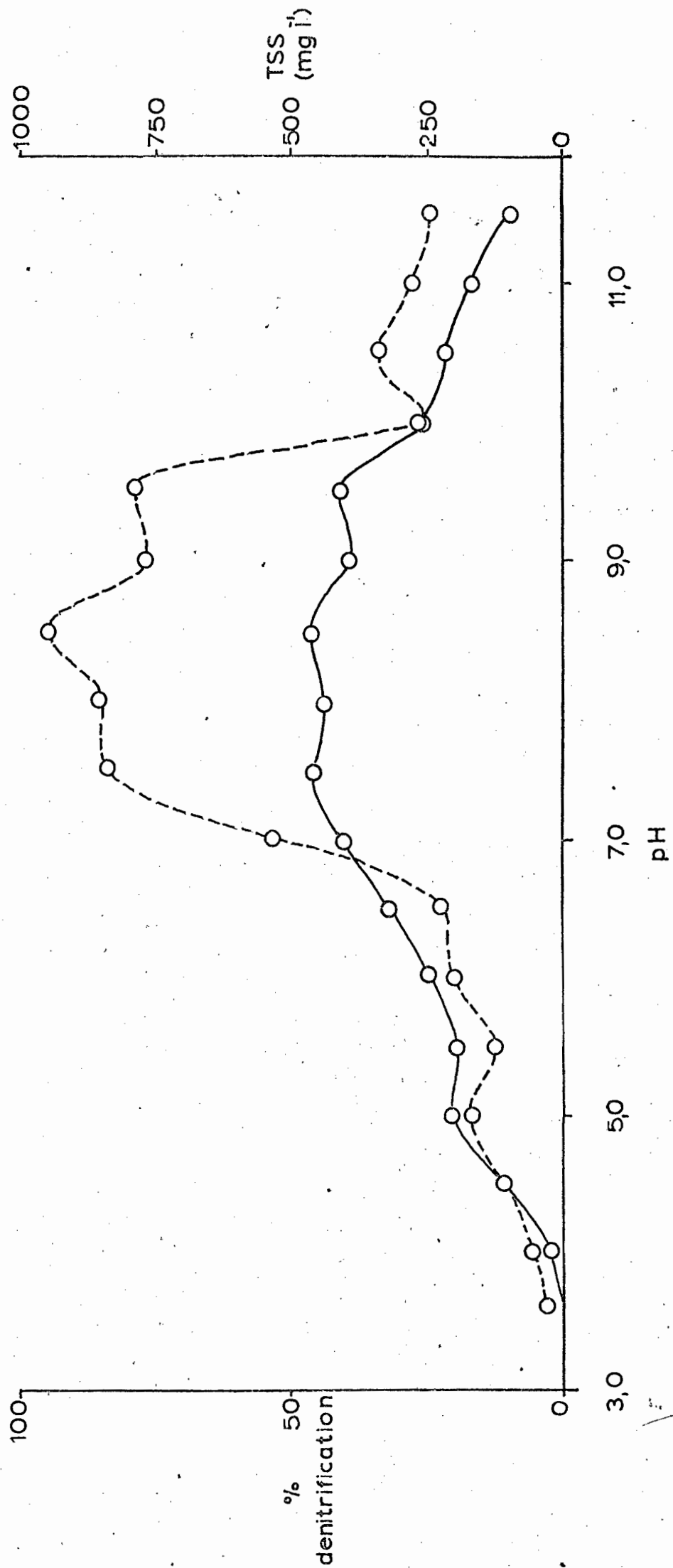






FIGURE 7 : Percentage denitrification and suspended solids at  
various pH values with methanol as the hydrogen donor

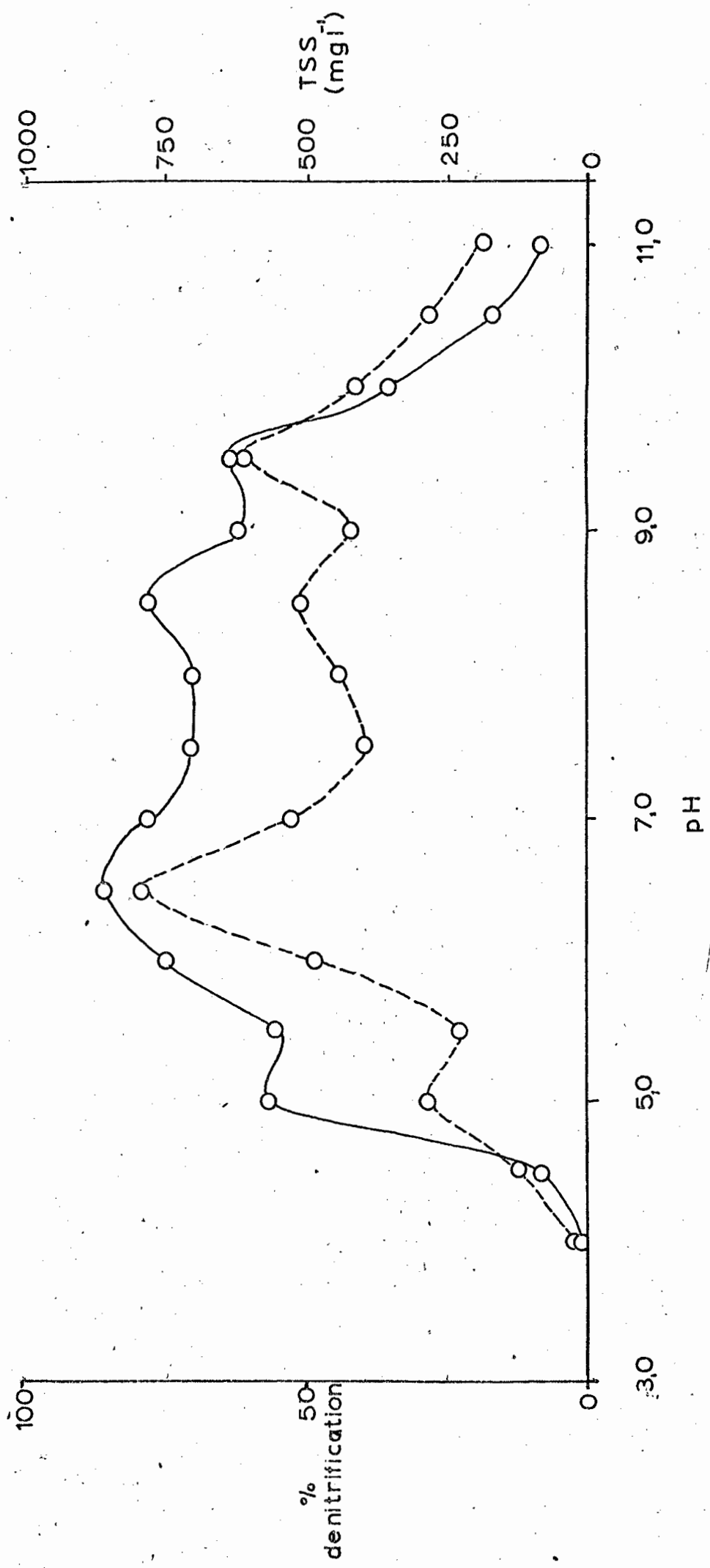




FIGURE 8 : Percentage denitrification and suspended solids at various pH values with lactate as the hydrogen donor

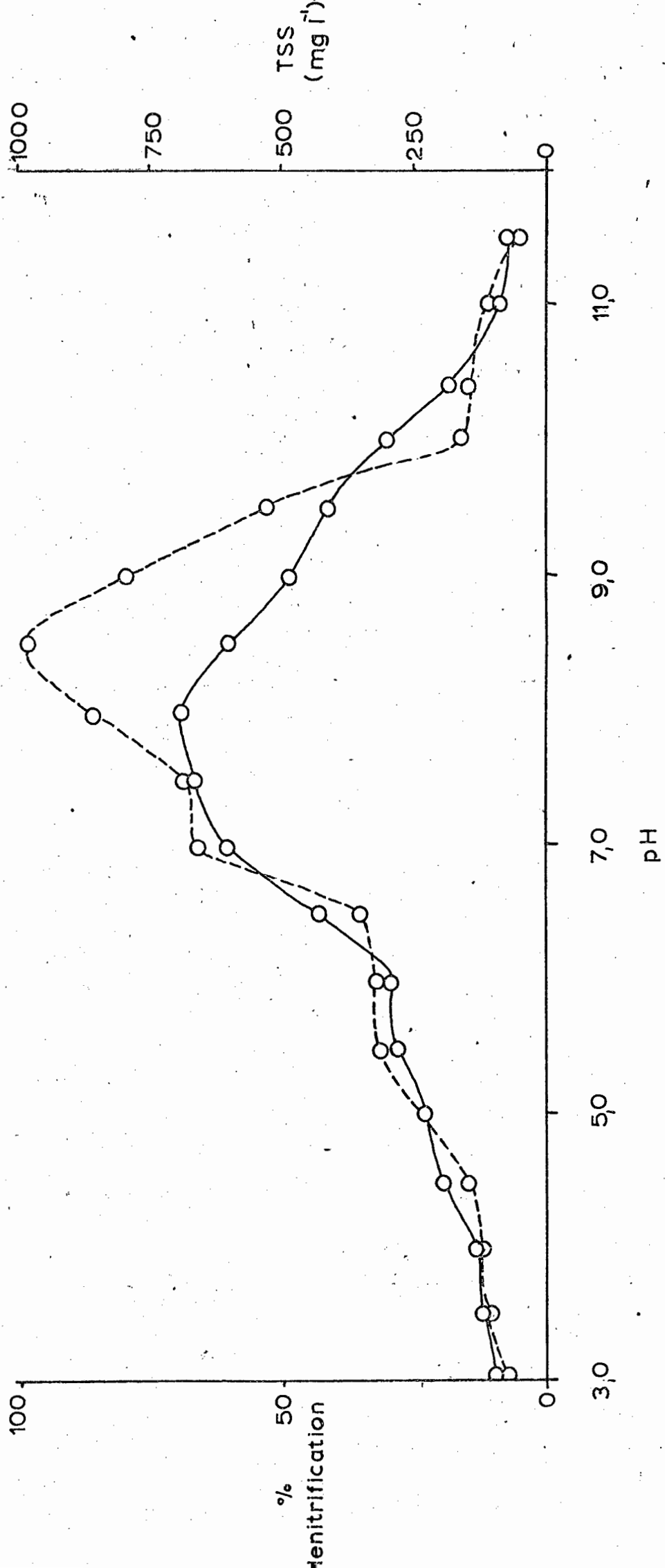


TABLE 9 : Cell production and denitrification by *Alcaligenes* sp. at different temperatures with a variety of carbon sources

Temperature	Methane		Methanol		Lactate	
	TSS (mg $\ell^{-1}$ )	% Denitrification	TSS (mg $\ell^{-1}$ )	% Denitrification	TSS (mg $\ell^{-1}$ )	% Denitrification
5°	151	3,9	115	11,8	-	-
10°	220	7,8	212	12,2	103	11,8
15°	424	24,0	299	25,3	111	24,8
20°	579	50,8	402	46,0	174	47,7
25°	636	49,9	534	58,6	322	57,9
30°	580	48,0	573	74,2	769	63,6
35°	441	37,8	526	72,7	872	71,6
40°	219	24,1	488	59,9	571	59,7
45°	150	16,9	407	46,5	485	21,6
50°	82	6,0	322	20,7	340	18,7
55°	43	2,1	252	12,2	303	15,1
60°	10	1,0	203	6,6	270	12,7
65°	-	-	-	-	230	11,0





FIGURE 9 : Percentage denitrification and suspended solids at  
various temperatures with methane as the hydrogen donor

Key to figures 9 - 11

————— Percentage denitrification  
----- Total suspended solids

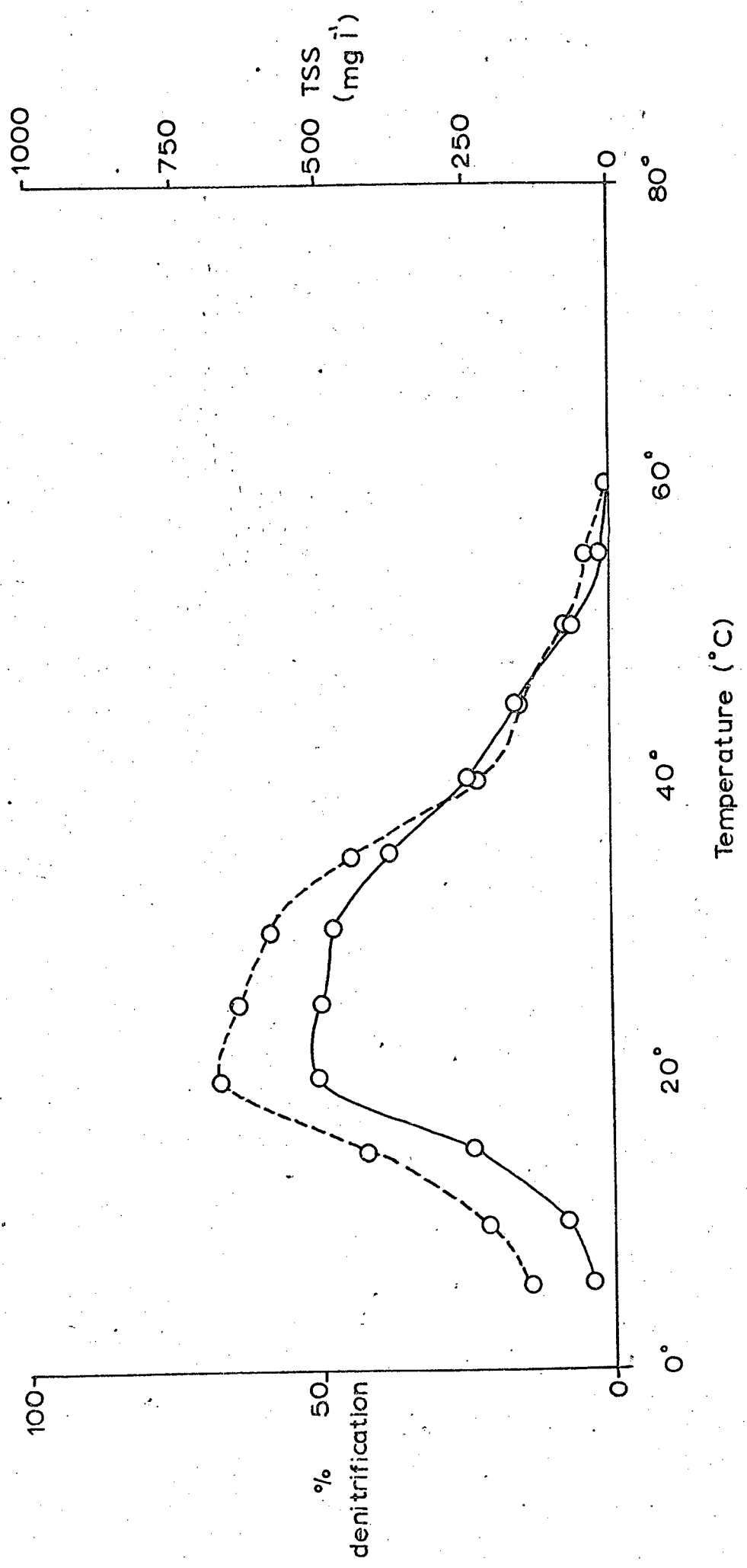




FIGURE 10 : Percentage denitrification and suspended solids at various temperatures with methanol as the hydrogen donor

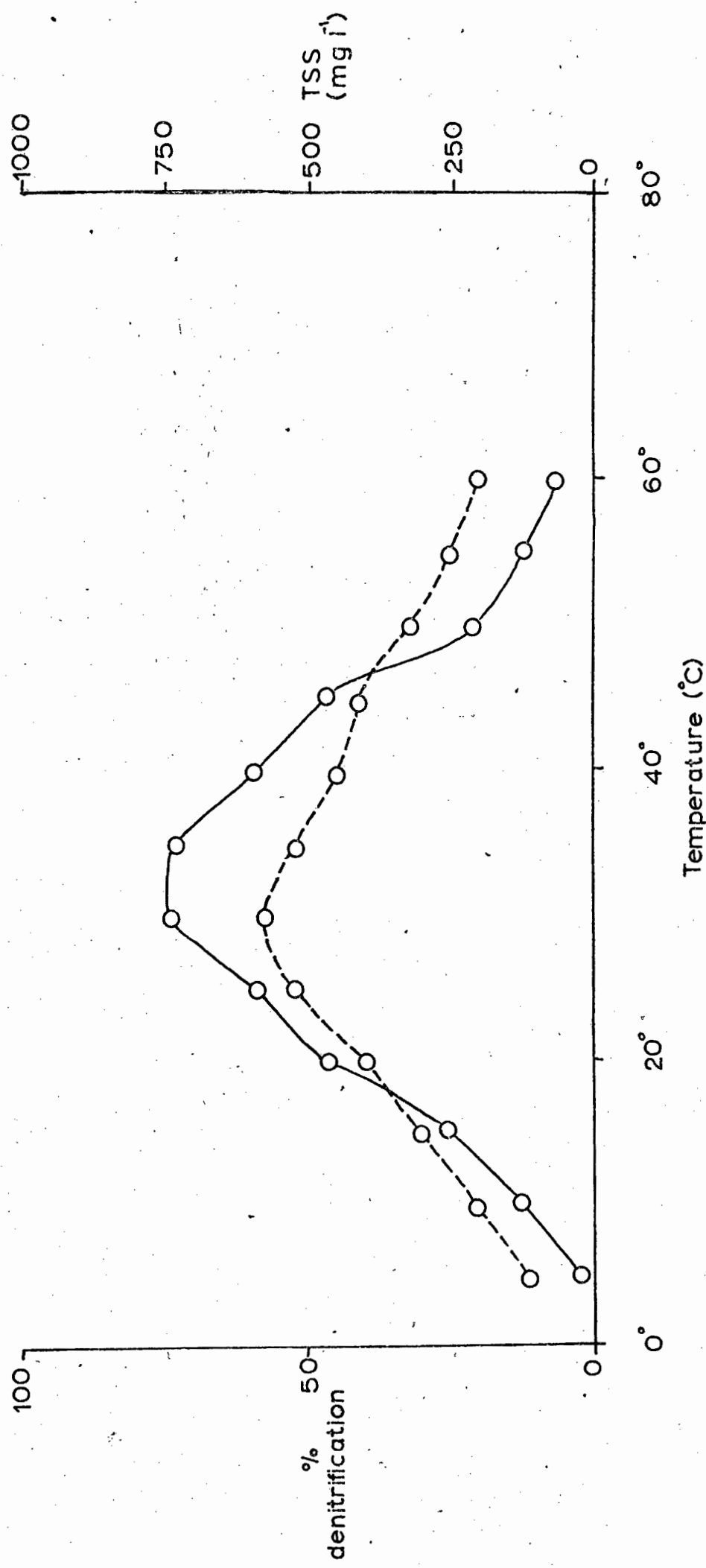
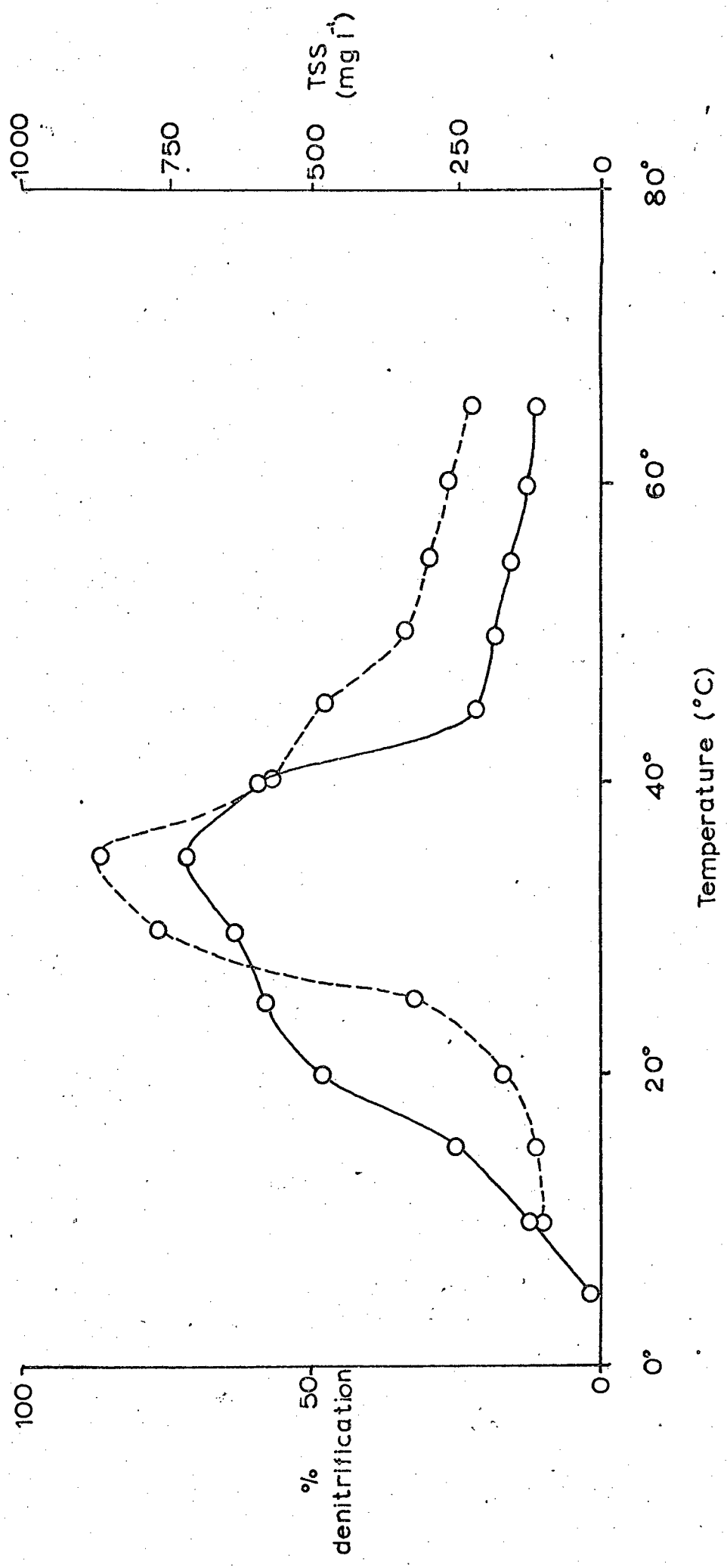




FIGURE 11 : Percentage denitrification and suspended solids at various temperatures with lactate as the hydrogen donor





culture, which flourished at temperatures between 20°C and 30°C. This may be due to methane becoming less soluble in water as the temperature increases.

A remarkable feature was that denitrification, although seriously retarded, was still recorded at temperatures as low as 5°C. At this temperature only about 3% denitrification was recorded over a ten hour period, compared with values approaching 80% at optimum temperature levels. It has been shown in these studies that at 10°C denitrification was as low as 10%. This however, was part of a steady decline in activity from about 25°C in the methanol and lactate cultures, while the decline began at 20°C in the methane culture.

This ability to grow and denitrify at low temperatures could, in the phraseology of Ingraham and Stokes (1959), be regarded as "facultative" psychrophilism as the bacterium was able to grow in the vicinity of 5°C, but exhibited optimum growth above 20°C. Farrell and Rose (1967) have concluded that such psychrophilism is a character unevenly spread among bacterial genera, but occurring chiefly among the Gram negative bacteria. Ingraham and Stokes were more specific, stating that "facultative" psychrophilism was a character possessed by members of both Alcaligenes and Achromobacter.

#### 4. DENITRIFYING UNIT WITH METHANE AS THE CARBON SOURCE

The packed column laboratory scale denitrifying unit receiving methane reduced the concentration of dissolved nitrate. A typical sample showed nitrogen concentrations reduced from 56,0 mg  $\ell^{-1}$  nitrate and 1,67 mg  $\ell^{-1}$  nitrite to 24,8 mg  $\ell^{-1}$  nitrate and 4,16 mg  $\ell^{-1}$  nitrite nitrogen. Although nitrite nitrogen concentrations rose there was, in fact a reduction in total nitrogen.

A further sample reduced dissolved nitrogen compounds from 56,1 mg  $\text{NO}_3\text{-N } \ell^{-1}$  and 2,35 mg  $\text{NO}_2\text{-N } \ell^{-1}$  to 29,65 mg  $\text{NO}_3\text{-N } \ell^{-1}$  and 2,63 mg  $\text{NO}_2\text{-N } \ell^{-1}$  in the effluent from the unit. This loss of dissolved nitrogen was balanced by the evolution of nitrogen shown by the differences in readings on the two gas-flow meters. This unit, then, receiving methane gas as a carbon source, was able to achieve denitrification values of the order of 50%.

This holds out great promise for the development of a denitrification system for water purification which will operate at virtually no cost.

## V. DISCUSSION

Settled domestic sewage is completely unsuitable as a carbon source for inducing denitrification. Although efficient C.O.D. removals can be obtained in the system, these removals are apparently unconnected with denitrification.

A further disadvantage of the use of settled sewage is the fact that it introduces large quantities of ammonia into the system which cannot be handled, and therefore the ammonia passes straight through.

Methanol is suitable for inducing an acceptable rate of denitrification. The 4:1 ratio of methanol to  $\text{NO}_3\text{-N}$  suggested by Barth et al. (1968) appears to be far too high if anaerobic conditions are maintained.

The bacteria capable of using this single carbon energy source are by no means dependent on methanol as a hydrogen donor, being able to use several completely unrelated organic carbon compounds as alternative hydrogen donors. This would simplify the development of a denitrifying system, as cell formation is relatively slower on methanol than on other carbon compounds. It should be possible to build up a sufficient population rapidly with some other carbon source, and then switch to methanol, the carbon of which is mostly evolved as  $\text{CO}_2$  during nitrate reduction.

Although  $\text{N}_2\text{O}$  production was partly dependent on the bacterial strain being used, a finding which agreed with those of Gayon and DuPetit (1882) and Cook (1963), it was also contingent on the nature of the carbon source. While Kluyver and Verhoeven (1954) described  $\text{N}_2\text{O}$  as a regular intermediate of denitrification, Naik and Nicholas (1965), Allen and Van Niel (1952) and Sacks and Barker (1953) all maintained that  $\text{N}_2\text{O}$  was produced by a mechanism different to that producing  $\text{N}_2$ . The findings of the present investigation tend to support the latter viewpoint.

Although Whittenbury et al. (1970) reported that the bacteria which they were investigating were unable to replace oxygen as a terminal electron acceptor during methane oxidation, this investigation has shown that other bacteria exist which can make use of nitrate respiration with methane as an electron donor. Denitrification has been achieved with methane as the sole electron donor in pure culture and in mixed cultures derived from settled sewage.

As nitrate was the only nitrogen source supplied in the culture media, the methane-utilising bacteria are certainly able to use nitrate nitrogen effectively for the synthesis of cell material, despite claims to the contrary (Vary and Johnson, 1967).

Several bacteria isolated from a denitrifying unit receiving methanol were able to utilise methane for denitrification. Bacteria isolated for their faculty to oxidise methane were able to grow and denitrify on methane, ethanol, malate or lactate. Both these observations serve to negate the theory of specificity of substrates among the methane-utilising bacteria. The explanation of this apparent disagreement with earlier reports of high specificity could lie in Foster and Davis' (1966) division of these bacteria into "obligate" and "facultative" methane utilisers. Any "obligate" methane utilisers must occur in very specialised habitats, as methane is normally not readily available. This would result in their being washed out of a unit such as a denitrifying column when carbon sources other than methane are being supplied. The relatively short time necessary to adapt a denitrifying unit to methane indicates that the vast majority of methane utilisers are already present and merely need to adapt their metabolism to a new carbon source.

The population of methane oxidising bacteria isolated from the methanol denitrifying unit and the population isolated from the methane unit were both dominated by the genus Alcaligenes. In Bergey's Manual

(7th edition, 1957) the generic characters of this genus are given as peritrichous or non-motile Gram negative rods. They do not normally ferment carbohydrates but they may or may not liquefy gelatin or solidified blood serum. They turn litmus milk alkaline and may peptonize it. They do not form acetylmethylcarbinol. Chromogenesis, when it occurs, is from greyish-yellow to brownish-yellow or yellow. They generally occur in the intestinal tract of vertebrates, dairy produce and water.

In general our knowledge of the physiology of Alcaligenes is very limited. Porter (1948) has stated that, although Alcaligenes is a commonly occurring genus, its fundamental nutritional requirements have not been closely studied. It has been grown at pH 7,8 on the following medium: 8 g NaCl; 1,5 g  $K_2HPO_4$ ; 0,25 g  $NH_4NO_3$ ; 0,5 g  $HgSO_4$ ; 2 g asparagine; 0,02 g cystine and 2,5 g sodium pyruvate, dissolved in one litre of distilled water.

No studies are apparently available on the kinds of nitrogen compounds which can be used.

Barron and Friedmann (1941) found one strain out of three of A. faecalis unable to oxidise glucose, concluding that this strain lacked phosphorylation ability. They reported complete oxidation of alcohols, amino acids, propionate and acetate, while lactate was 97% oxidised, propionate 92% and butyrate 80,4%.

Tulukova (1959) suggested that the genus Alcaligenes might merely contain variations of other enteric bacteria, as Escherichia coli and Salmonella typhi can both be induced to produce alkali. Of the natural alkali producers studied, 20% showed antigenic groupings common to artificially induced alkali producers.

Vary and Johnson (1967) found that, although isolates had a shorter generation time when grown aerobically with methanol, greater cell weight was eventually produced by cultures grown on methane. This corresponds with the results of Whittenbury et al. (1970), whose cultures yielded 20% more dry weight with methane than with methanol, and is fully in agreement with the results obtained in the present investigation.

The heterotrophic oxidation of methane reported here validates the implied suggestion of Hansen and Kallio (1957), who excluded methane when they observed that the anaerobic oxidation of hydrocarbons by nitrate-reducing bacteria was an infrequent occurrence.

Denitrification was not as good when methane was used as the hydrogen donor as with other carbon compounds, as a relatively large proportion of the nitrate nitrogen went into cell nitrogen, causing high TSS values. Denitrification rates never rose above 50%, but they were nevertheless substantial enough to suggest that methane could be used in commercial systems, if operated with enough recycling to reduce load on the denitrifying unit.

It is apparent that the bacteria able to utilise methane for denitrification, are metabolically most efficient in the range 30 - 35°C, except when methane itself is the hydrogen donor, when maximum growth and denitrification were obtained between 20° and 30°C. Although efficiency is detrimentally affected when the temperature drops below 10°C, the bacteria were able to grow at a reduced rate at these low temperatures. Although cold winter temperatures would make a denitrifying system less effective, the bacteria should be able to recover from these colder periods without a long lag period.

Fortunately the optimum growth and denitrification range was slightly alkaline. The pH tends to increase during denitrification, but effluents never contain enough nitrate to make the pH pass out of the optimum operating range.

This investigation has shown that methane could be an ideal carbon source in commercial denitrifying units. If hydrogen donors were to be added in a liquid or solid form the concentration of nitrate in the water would have to be monitored in order to ensure that the correct C:N ratio was maintained. Load fluctuations could upset such a system and reduce its efficiency. The addition of too little carbon would lead to incomplete nitrogen removal, too much would leave a residual C.O.D. and constitute further pollution. The use of a gaseous hydrogen donor would circumvent these complications.

As methane is only about one tenth as soluble in water as oxygen, this could produce some problems. A modification of the revolving disc unit (Pretorius, 1971) to enclose the discs and provide them with an atmosphere of gas effluent from an anaerobic digester could solve the gas solution problem, however. It would also act to retain cells formed during treatment, as methane appears to induce the production of relatively large cell masses in relation to the amount of nitrogen gas produced.

The use of methane could overcome most of the objections to the use of denitrification in water purification schemes. It would, in particular, overcome the familiar objection that there should be sufficient carbon present at sewage treatment plants to achieve any denitrification necessary.

These findings could dramatically change previous approaches to water reclamation by drastically cutting the costs of such processes.

## VI. CONCLUSIONS

1. Settled sewage was not suitable as a source of hydrogen donors for denitrification.
2. Methanol was suitable for denitrification, but the 4:1 ratio for C:N of Barth et al. (1968) was far too high.
3. Bacteria using methanol for denitrification were not specific to methanol.
4.  $N_2O$  production is dependent on both bacterial strain and the nature of the carbon source.
5. Denitrification is possible with methane as a carbon source.
6. The bacteria responsible for this reaction were ordinary denitrifying bacteria, which were in no way specific to  $C_1$  compounds.
7. Alcaligenes was the predominant genus among methane oxidising bacteria isolated from both methanol and methane denitrifying units.
8. No isolates were found to be able to denitrify using molecular hydrogen for nitrate reduction.
9. The optimum pH range for denitrification by these isolates was 7,0 to 8,5 when denitrification values approached 80%. This fell off sharply below pH 6,5 or above 9,0, where denitrification was closer to 25% or less.
10. The pH did not have any effect on the nature of the nitrogenous gases produced as reduction products.



11. Denitrification and growth rates were drastically affected by temperature. At 30°C denitrification was of the order of 80% in the methanol and lactate cultures, while at 5°C growth was still recorded, but denitrification fell to 3% over a ten hour residence time. In the methane culture the bacteria grew most efficiently between 20°C and 30°C.
12. Denitrifying efficiency of 50% was obtained in a packed column unit using methane as the carbon source.
13. Methane holds great promise as a hydrogen donor for commercial denitrification systems.

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